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XXV CICLE

PhD THESIS

THE SALIVA PRECIPITATION INDEX (SPI): AN INNOVATIVE ANALYTICAL METHOD TO MEASURE WINE ASTRINGENCY

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la mia famiglia

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As one of life's finest pleasures, the alluring aroma, the desirable taste, and the typical color of red wines have been attracting consumers for more than 2000 years.

Preface

Quality of food is not easy to define since different working definitions can be formulated depending upon who is defining them and the level at which it is measured. One popular definition comes from a USDA Marketing Workshop Report cited by Gould (1977). It reads "the combination of attributes or characteristics of a product that have significance in determining the degree of acceptability of the product to a user". Introducing the definition of the sensory quality, Galvez and Resurrection (1992) term as: "the acceptance of the perceived characteristics of a product by consumers who are the regular users of the product category or those who comprise the target market". The way we perceive the characteristics of a food as well as beverage is fundamental not only for its conceptual quality but also for its acceptance. Steenkamp's (1989) concept of perceived quality attempts to mediate between objective product characteristics and consumer preferences. It stresses that perceived quality may differ from objective quality because individual assessments of quality are personal and situational (Holm & Kildevang, 1996). Since perceived quality is an abstract construct and is of a multi-dimensional nature, the development of mesuration tool is a question of vital importance, for both researchers and practitioners.

Astringency is an important sensory characteristic of food and beverages containing polyphenols. This mouthfeel is mainly due to the interactions of polyphenols with salivary proteins, causing complexes formation and their further precipitation, which leads to a reduction of the lubricating properties of saliva. As a consequence, sensations of dryness, hardness, and constriction are felt in the mouth. Wine astringency is generally estimated by tasting, which shows some difficulties in discerning among tastes, is labourious and time-spending and may be restricted to a limited number of samples.

A method that is more objective as possible and that takes into account the multiperceptual phenomenon of astringency is the desired goal for many researchers. In addition, the possibility to evaluate the influence of both grape and wine phenolics, as well as factors and enological practices on astringency is a great challenge also for industry to manage wine quality.

Prefazione

La qualità di un alimento non è semplice da definire in quanto la opportuna formulazione di tale concetto dipende sia dal soggetto che lo definisce sia dal metodo di misura utilizzato per valutarlo. Una delle definizioni più note è quella riportata da Gould (1977) nel report del USDA Marketing Workshop: "la combinazione di attributi e caratteristiche di un prodotto che hanno importanza nel determinare il grado di accettabilità del prodotto da parte del consumatore". In seguito, introducendo il concetto di percezione sensoriale, Galvez and Resurrection (1992), hanno dato la seguente definizione di qualità: "l'accettabilità delle caratteristiche percepite di un prodotto dal consumatore che è un utilizzatore regolare di quella categoria di prodotto o di quelle che comprendono la stessa fascia di mercato". Il modo con cui percepiamo le caratteristiche di un cibo o di una bevanda è fondamentale non solo per la sua qualità intrinseca ma anche per la sua accettabilità. Il concetto di qualità sensoriale di Steenkamp (1989) cerca di mediare tra le caratteristiche oggettive del prodotto e le preferenze del consumatore. Esso afferma che la qualità percepita può differire da quella oggettiva perché le valutazioni individuali della qualità sono personali e situazionali (Holm & Kildevang, 1996). Dato che la qualità percepita è un costrutto astratto ed è di natura multi-dimensionale, lo sviluppo di un metodo di misurazione è di fondamentale importanza, sia per i ricercatori che per i praticanti.

L'astringenza è una importante caratteristica sensoriale di alimenti e bevande contenenti polifenoli. Questa sensazione tattile è dovuta principalmente alla precipitazione delle proteine salivari da parte dei polifenoli con derivante riduzione delle proprietà lubrificanti della saliva. Come conseguenza sensazioni di secchezza, durezza e costrizione vengono avvertite in bocca.

L'astringenza del vino viene generalmente valutata attraverso la degustazione, che però presenta alcune problematiche come la difficoltà di distinguerla dagli altri gusti, la laboriosità e la necessità di molto tempo per l'addestramento dei giudici, inoltre soltanto un ristretto numero di campioni possono essere valutati contemporaneamente. Un metodo che sia il più oggettivo possibile e che tiene in conto il fenomeno multi-percettivo dell'astringenza, è un obiettivo che molti ricercatori desiderano raggiungere. Inoltre, la possibilità di valutare l'influenza che i fenoli sia dell'uva che del vino, così come i fattori e le pratiche enologiche, hanno sull'astringenza è una grande sfida anche per l'industria per poter meglio gestire la qualità del vino.

Introduction

Wine quality ideally should be related to intrinsic visual, taste, or aroma characters which are perceived as above average for that type of wine. In particular, the gustative balance is fundamental in defining the quality of a red wine that is determined by a fine equilibrium among three components: sweetness, acidity and astringency.
Astringency is a tactile sensation evoked in mouth by plant polyphenols-derived products, such as red wine. It is felt as the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins" (ASTM, 1991).
Astringency may be considered as a negative attribute by the consumer if wines were perceived as harsh and rough, because the gustative balance is shifted toward the tannic component. If a good compromise between phenolic quantity and quality was achieved it can enhance the complexity and palate length during tasting. As additional values, long-aging potential and antioxidant capacity of wine depend on the phenolics profiling. Therefore, although phenolics are useful to prolong wine shelf-life, they can also determine an excessive astringency.
An objective tool able to measure astringency is not still available. The evaluation of astringency by tasting is not easy to achieve since this sensation is subject to a certain subjectivity and is difficult to discern. For this reason chemical evaluation of astringency shows a high potential of interest both for researchers and winemakers.

In this project the focus of the literature review is on the instrumental methods that have been developed to assess wine astringency during last decades. It provides an overview of the research concerned with elucidating the definition, perception, physiology and mechanism of astringency, the interactions between saliva and polyphenols, the *pro et contra* of different methods and the correlation between instrumental and sensory data.
In the thesis work an innovative method based on the precipitation of human saliva (Saliva Precipitation Index, SPI) with grape and wine polyphenols has been developed in order to measure astringency.

The principal aim of this thesis is to develop and improve an analytical method able to measure wine astringency based on the physiological phenomenon that happens during tasting, the precipitation of salivary proteins by polyphenols. The method based on the SDS-PAGE of human saliva furnishes and index named saliva precipitation index (SPI) able to evaluate the reactivity of polyphenols toward selected salivary proteins.
An ulterior aim is the application of the SPI to evaluate factors as different *stimuli* and wine constituents, and enological practices influencing astringency. The focus was on the analysis of human saliva by the SDS-PAGE electrophoresis and on the reactivity of some salivary proteins with polyphenols. Used materials were commercial tannins, as well as grape extracts and wines. The special aim was to study how wine components as pH, ethanol, mannoproteins and fructose influenced the binding with salivary proteins and then the sensory perception of wine. Additionally, the effects of dealcoholisation and fining of wine on the SPI and sensory profiles were studied. One special focus was the study of the characterization of Aglianico (*Vitis Vinifera* L. cv) grapes phenolics by HPLC-MS and the correlation of the structural characteristics with SPI in order to better define oral astringency.

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"There are no applied sciences....There are only applications of Science and this is a very different matter....The study of the applications of Science is very easy to anyone who is master of the theory of it"

(Louis Pasteur)

Chapter One

Literature Review

1.1 Astringency

1.1.1 Definition of astringency

The term astringency derives from the Latin verb *a-strigo (ad-stringo), strinxi, strictum, a-stringere* that means tightly bind, strongly join. It refers to the propensity of vegetable tannins to complex with macromolecules, such as proteins and polysaccharides, and alckaloids. This ability to combine with proteins is the basis of the process known as vegetable tannage, by which an animal skin is converted to leather.

The chemical definition of astringency is solely related to the ability to bind proteins, whereas it is often described in sensory terms as a roughing, drying or puckering sensation in the mouth (ASTM, 2004). Bate-Smith (1954) first speculated that astringent sensations were caused by the increase in friction between the mucosal surfaces which resulted from reduction in lubrication as salivary proteins were bound by astringent compounds. Often confused with bitterness, which is the sensation perceived at the back of the tongue, astringency was also defined as the "dry-mouth'' feeling thought to be produced by the interaction of polyphenols with the proteins of the mouth (Singleton and Esau, 1969). Differently from tastes as bitterness, astringency is a mouthfeel sensation felt in the oral cavity after the ingestion of food and beverages containing polyphenols due to the precipitation of salivary proteins by polyphenols, which determines a lack of lubricity and an increased friction between mouth surfaces. Astringency then can be defined as a tactile sensation, because: (i) it is perceived on non-gustatory surfaces such as on soft palate, gingives, lips, (ii) does not show adaptation but also (iii) increases upon repeated ingestion.

1.1.2 Astringency sub-qualities

The soluble complexes and precipitates formed by polyphenols and salivary proteins stimulated and activated mechanoreceptors (MRs) hold in mouth. MRs are nerve endings that function like those of the skin, except that they have smaller receptive fields and lower activation thresholds (Trulsson and Essick, 1997). They differ in structure and distribution in the oral cavity and in their response to static and dynamic mechanical events. Oral MRs are selectively sensitive to different *stimulus* properties, such as particle size and/or mouth movements, and project such information to the central nervous system (Chen and Engelen, 2012). The sensitivity of MRs to astringents may explain astringency as a multiple perceptual phenomenon. Generally, it has been described as a combination of three sensations as drying, puckering and roughing. These were defined as (i) 'drying', the lack of lubrication or moistness resulting in friction between oral surfaces, (ii) 'roughing', un-smooth texture in the oral cavity marked by inequalities, ridges and/or

projections felt when oral surfaces come in contact with one another, (iii) 'puckery', drawing or tightening sensation felt in the mouth, lips and/or cheeks, and (iv) 'astringency' being the complex of the other three (Lawless *et al*., 1994). Other terms such as 'stickiness' (defined as the sensation of the tongue adhering to the palate), 'oily mouthcoat' (slippery oil-like film), 'gritty' (the feeling of minute rough granules) and 'powdery' have been used by tasters to describe beer astringency (Meilgaard and Muller 1987, Langstaff *et al*. 1991). Peynaud (1987) also gives a list of astringent qualities in wine including 'noble', 'vegetal', 'bitter', 'acidic', 'harsh' and 'woody'. Gawel *et al*. (1998) derived 21 concrete terms such as 'silk', 'emery paper' and 'chalky', as well as 14 abstract descriptors to represent the astringency elicited by red wines. More recently, a wide lexicon ("mouth-feel wheel") was developed to assist in identifying and classifying a wide range of oral sensations elicited by red wine, which included 33 terms or sub-qualities to define astringency (Gawel *et al*., 2001). Among these harsh, unripe, dynamic, and drying have been found to define astringency as a negative sensation, while the complex and mouthcoat qualities have been associated to a positive impact. These sub-qualities were also associated with touch standards when utilized to describe the tactile astringent sensations in the mouth elicited by red wines (De Miglio and Pickering, 2008). The wide vocabulary that characterize astringency, reveals that this tactile sensation is not easy to define and that the structural composition of an astringent compound may determine a different reactivity towards salivary proteins and then a different perception in mouth.

1.1.3 Mechanism of perception

The perception of astringency is basically a dynamic process, that continuously changes and evolves to reach a maximum of intensity once red wine is ingested. Following the physiological course, what happens in mouth during wine tasting? When a sip of wine is made, a volume of wine is introduced in the oral cavity. According to sensory protocols, it is necessary to hold wine in the mouth for almost 8 s, during which wine constituents mix with saliva at a temperature of 37 °C. After this time, wine can be expectorated or ingested together with saliva. Astringency takes many seconds (15 s) to develop fully (Kallithraka *et al.,* 2001). As a dynamic sensation, astringency takes time to develop fully. TDS sensory studies on wine stated that after the sweet, sour and bitter tastes, at about 35 sec after expectoration or ingestion, astringency takes place (Pessina *et al.,* 2004). The time dependent evolution of this sensation is consistent with the mechanism of perception proposed by Jöbstl *et al*. in 2004 in which three stages were described: (i) the simultaneous binding of the multidentate polyphenols to several sites on the free protein whose structure evolves from a loose and extended conformation to a more compact one; (ii) as the polyphenol concentration rises, polyphenols cross-link different protein molecules leading to dimerization and (iii) aggregation into larger particles that finally precipitate.

1.1.4 *Stimuli*

Compounds able to elicit sensations as tastes and mouthfeels are called *stimuli*. Once introduced in mouth these compounds activated taste transduction pathways if they related to sweet, sour, salty and bitter tastes or mechanoreceptors for tactile sensations if the substances are astringent. Astringents commonly defined are tannins, that can be classified in condensed tannins, phlorotannins and ellagitannins. Chemically diverse astringents such as complex salts such as aluminum sulfate (alum), acids and other phenolics, have been also shown to evoke astringency (Lee and Lawless 1991, Lawless *et al.* 1994).

1.1.4.1 Tannins

Wines that are astringent are in common parlance termed “tannic”, as tannins are the main polyphenolic compounds involved in the sensation. Swain and Bate-Smith (1962) provided the first useful phytochemical definition of tannin, being "water-soluble phenolic compounds, having molecular weights lying between 500 and 3000, which have the ability to precipitate alkaloids, gelatin and other proteins".

Tannin is a generic term gathering 3 groups of structurally different molecules: phlorotannins, condensed tannins, and hydrolysable tannins. Phlorotannins are found mainly in marine organisms such as brown algae (Arnold and Targett, 2002) and they are structurally perhaps the most simple tannin group. Individual phlorotannins are composed of two or more phloroglucinol (Fig. 1a) units that are attached to each other via C-C or C-O-C bonds, thus yielding oligomers such as the tetrameric phlorotannin (Fig. 1b). Further structural variations may include additional OH-groups in the molecules or additional bonds between the monomers. This structure-based definition very clearly differentiates phlorotannins from other types of phenolic compounds.

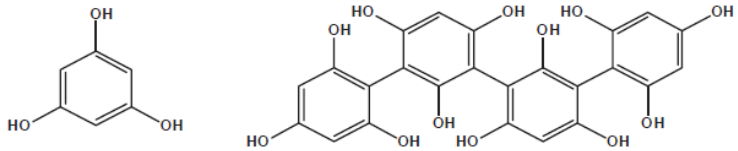

Fig. 1: Structure of phloroglucinol (a), the phlorotannin unit, and a tetrameric phlorotannin (b) consisting of four phlotoglucinol units.

Condensed tannins represents a large majority of tannins present in grapes and wines. They are polymers with more than three units composed of terminal and extension subunits analogous to the flavan-3-ols catechin, epicatechin, epicatechin-gallate, and epigallocatechin (Fig. 2). In a hot and acid medium the interflavanic bond breaks down releasing an unstable carbocation producing, eventually, an anthocyanin. For this reason flavan-3-ol polymers are also called proanthocyanidin (Bate-Smith, 1975).

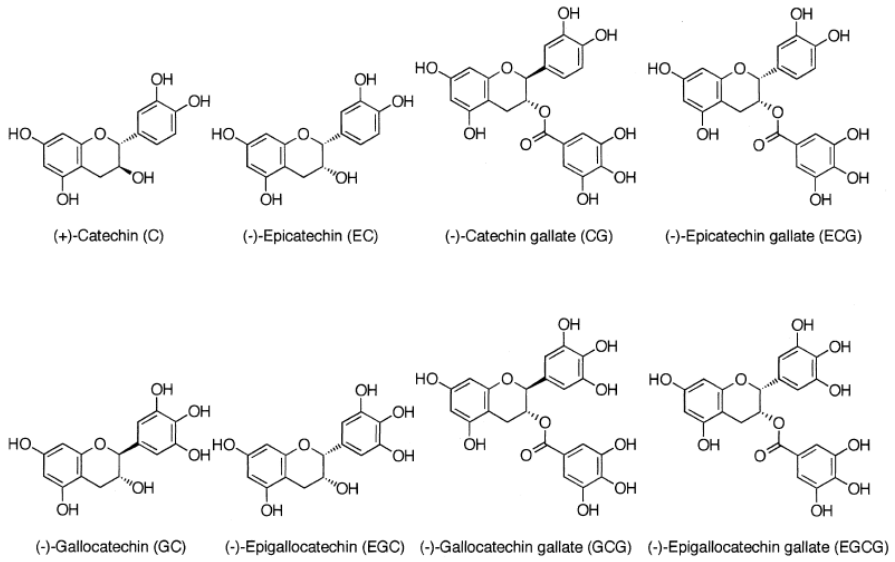

Fig. 2: Common monomeric units of condensed tannins.

More specifically, procyanidins produce cyanidin from catechin and epicatechin, while prodelphinidins produce delphinidin from gallocatechin and epigallocatechin. Proanthocyanidins (examples in Fig. 3) can be classified on the basis of the kind of chemical bond:

- ✓ Proanthocyanidins A ($C_{30}H_{24}O_{12}$): dimer proanthocyanidins with two flavan-3-ols condensed with a C4-C6 or a C4-C8 bond (interflavanic bond) and forming an ether between the C2 of the first unit and the C5 and C7 of the terminal unit.
- ✓ Proanthocyanidins B ($C_{30}H_{26}O_{12}$): dimer proanthocyanidins with only a C4-C6 or C4-C8 interflavanic bond.
- ✓ Proanthocyanidins C: trimer proanthocyanidins with only a C4-C6 or C4-C8 interflavanic bond.
- ✓ Proanthocyanidins D: trimer proanthocyanidins. In this case the first two monomers have the interflavanic bond only, but the central and the terminal monomer have both the interflavanic and the ether bond.

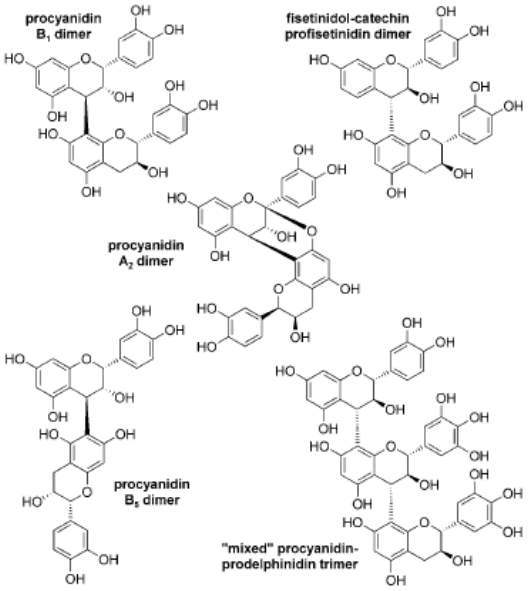

Fig. 3: Examlpes of condensed tannins**.**

Condensed tannins, whose composition is listed in Tab. 1, are extracted from grape seeds, skins, and stems during the winemaking process (Ribéreau-Gayon, 1974).

Tab. 1: Composition of skins, seeds and stems tannins (from Souquet *et al*., 2000).

TANNINS		
SKINS	**SEEDS**	**STEMS**
(+) catechin	(+) catechin	(+) catechin
(-) epicatechin	(-) epicatechin	(-) epicatechin
(-)epicatechin-3-O-gallate	(-)epicatechin-3-O-gallate	(-)epicatechin-3-O-gallate
(-) epigallocatechin		(-) epigallocatechin
mDP* = 30	mDP = 10	mDP = 9
Galloylation = 3-10%	Galloylation = 20-40%	Galloylation = 15-20%
High mDP	Oligomers=60%	High mDP

* mDP= mean degree of polymerization

Mouth-feel properties of grape seed and skin proanthocyanidins have been examined through many studies (Robichaud and Noble, 2006; Brossaud *et al.,* 2008). A study showed that astringency increased with the degree of polymerization and that an increasing degree of galloylation was responsible for an increasing coarse perception of the proanthocyanidins (Vidal *et al.,* 2003). Increasing the degree of B-ring trihydroxylation (given by epigallocatechin content) seemed to decrease astringency.
Generally, as the degree of polymerization increases, the astringency of the compounds increases. Oligomers are more astringent than monomers, but less bitter. Monomer epicatechin has been reported as more astringent than catechin (Noble, 1994; Kallithraka *et al.,* 1997). The astringency induced by purified grape compounds such as the monomeric flavan-3-ols, is quite different from that elicited by the total grape extracts and by mixtures of phenolic compounds such as tannic acid or hydrolysable tannins. In addition, the influence of these compounds in wine depends on many factors such as the ratio between the aggressive tannins/soft tannins, grape variety, growing conditions,

climate and winemaking practices, comprised the utilization of wood tannins and oxygen management.
Another class of tannin present in wine derived from the use of wood barrels, wood chips or oenological tannins (Ribéreau-Gayon 1972, Saucier *et al.*, 2006) is represented by hydrolyzable tannins (Fig.4). Hydrolysable tannins include gallotannins and ellagitannins.

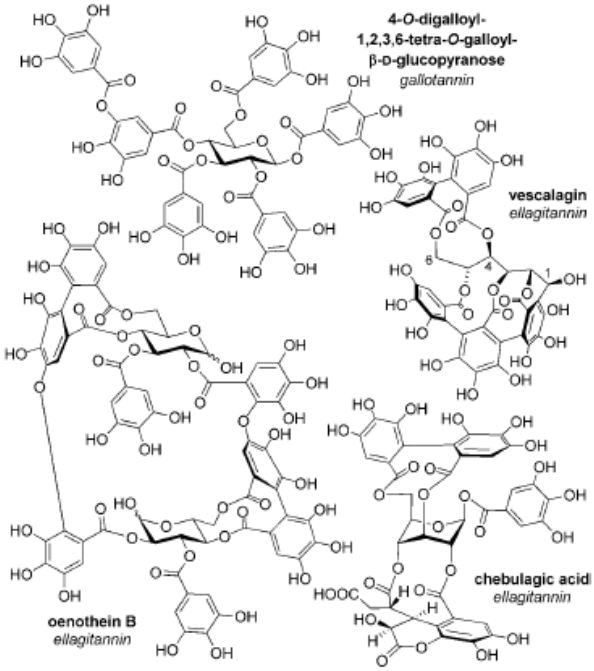

Fig. 4: Examples of hydrolizable tannins**.**

Both are derived from galloyl units [(a), Fig.5] esterified to a sugar core (Quideau and Feldman, 1997). When one [monogalloylglucose (b), Fig.5] to five [pentagalloylglucose, (d) Fig.5] galloyl groups are attached to the central moiety, they are commonly called as simple gallic acid derivatives. If six or more galloyl groups are in the structure, the compounds are called gallotannins and have one or more digalloyl groups [(e) Fig.5]. The most common and complex hydrolysable tannins in plants are ellagitannins (ETs). ETs are esters of hexahydroxydiphenic acid and a polyol, usually glucose or quinic acid [(b) and (c) in Fig.5]. When exposed to acids or bases, ester bonds are hydrolysed and the hexahydroxydiphenic acid spontaneously rearranges into the water-insoluble ellagic acid (EA), hence their name. ETs very likely derive from a common gallotannin biosynthetic precursor, penta-O-galloyl-β-D-glucose, by the oxidative formation of one or several biphenyl bonds between two or more galloyl residues.

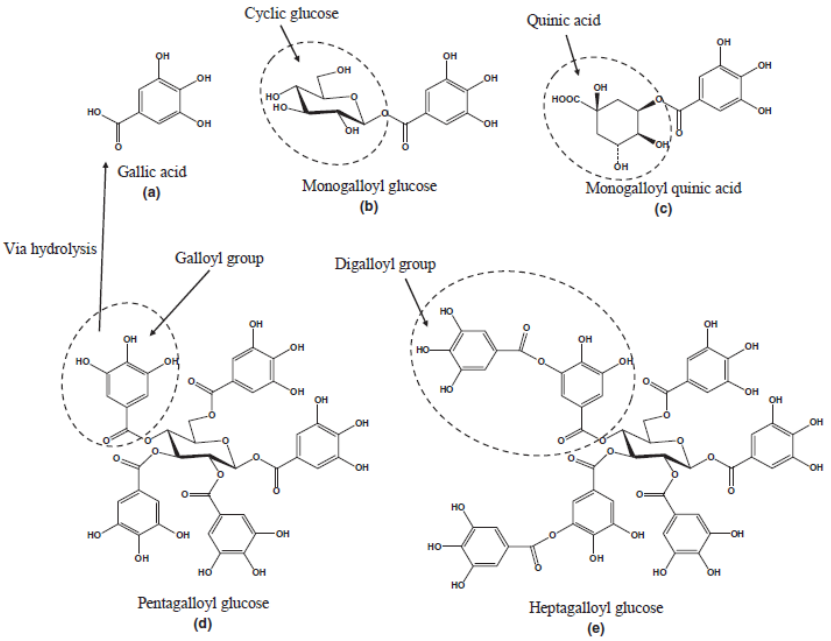

Fig. 5: Examples of the structures of simple acid gallic derivates (b-d) and gallotannin (e).

ET monomers can be further oxidised in plants and form dimers, trimers and tetramers with molecular weights up to 4000. Ellagitannin monomers, such as grandinin and roburin E were found to mainly contribute in to red wine astringency, and with less extend vescalagin and castalagin too. The threshold concentrations for astringency of the dimers roburin B and C were significantly high, meaning that the glycosidic moiety may interfere with the interaction with salivary proteins (Glabasnia and Hofmann, 2006). Using the half-mouth test, the perceived astringencies of oak wood ellagitannins were associated to smooth and mouthdrying sensations at low concentrations (Stark *et al*., 2010), Flavano-ellagitannins, such as acutissimins A and B (4a and 4b) and epiacutissimins A and B (5a and 5 b) (Fig. 6), were perceived as smooth and in higher concentrations as puckering. However, in oak wood matured wines they were found in low amounts, indicating a less significant contribution to red wine astringency (Stark *et al*., 2010).

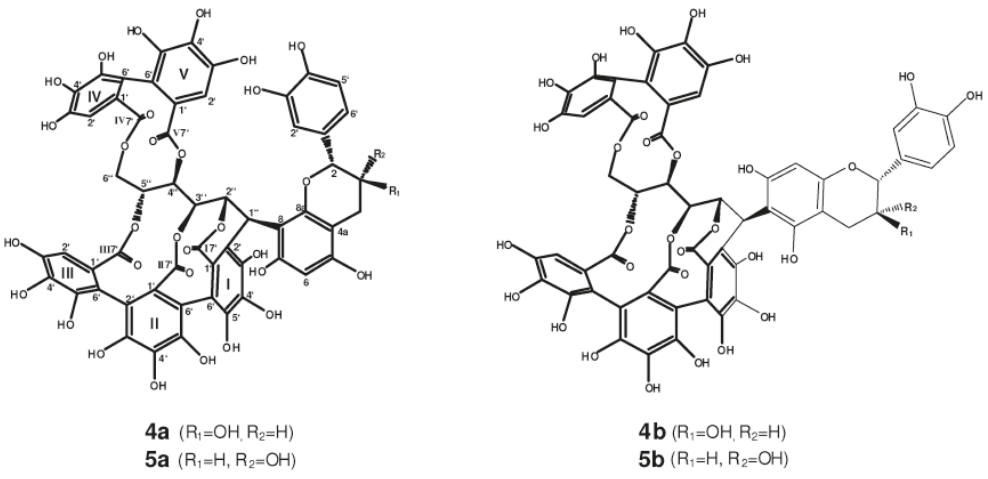

Fig.6: Chemical structures of acutissimin A (4a) and B (4b) and of epiacutissimin A (5a) and B (5b).

Tannic acid, commonly used as a reference compound for astringency in sensory studies, is a mixture of gallotannins with impurities, such as gallic acid and various gallic acid derivatives (Salminen and Karonen, 2011). It showed a high protein-precipitation capacity and a high impact on astringency sensation. The structure of tannic and gallic acid are shown in Fig.7.

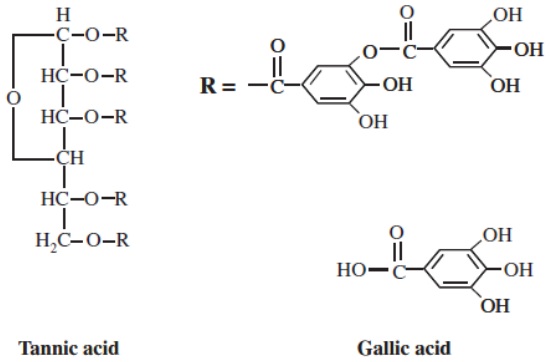

Fig. 7: Structure of tannic acid and gallic acid.

1.1.4.2 Acids

Although sourness is the predominant sensation of organic acids, dryness or astringency of acids has also been reported (Lee and Lawless, 1991; Rubico and McDaniel, 1992). Astringency of acids is attributed either to the direct contribution of H^+ ions or to the hydrogen bonding capabilities of the hydroxyl groups on the anion or undissociated acid (Lawless *et al.*, 1994). Five organic acids and one inorganic elicited astringency and astringent subqualities (Thomas and Lawless, 1995). In order to explain the sensory properties of organic acids they suggested that acids without hydroxyl groups might act by other mechanisms such as the denaturation of proteins in the saliva or direct attack on the mucous layer and oral epithelium.

1.1.4.3 Other phenolic compounds

Besides flavan-3-ols, the building blocks of grape tannins, anthocyanins, which impart color to the grapes and red wine, and flavonols are also present in grapes. There are five anthocyanidins (cyanidin, peonidin, delphinidin, petunidin, and malvidin) in grapes. Anthocyanin is a glycosylated anthocyanidin (sugar bound to the anthocyanidin moiety). Anthocyanins impart red/purple/black color in grapes. These base compounds also are found acylated (acid linked to the 6^{th} position of the sugar) with acetic, coumaric and caffeic acids, making them more stable. At the pH of wine, about 10% of wine anthocyanins are in colored form. The extraction and management of anthocyanins in young wines is vital to red wine quality and style, as evidenced by the positive correlations between red wine colour and overall wine quality (Somers and Evans, 1974; Mazza and Francis, 1995). Finally, flavonols (kaempferol, quercetin, and myricetin) are present in grapes and wine as glycosides (sugar attached). Flavonols are important cofactors for color enhancement. They also act as a natural sunscreen in the skin of grape berries. It has been shown that free anthocyanins, like the coloured tannin-like polyphenolic compounds from wine and pomace, do not contribute astringency nor bitterness to wine (Vidal *et al*., 2004).

Recently, a number of 26 sensory active nonvolatiles comprising hydroxybenzoic acids, hydroxycinnamic acids, flavon-3-ol glycosides, and dihydroflavon-3-ol rhamnosides as well as a structurally undefined polymeric fraction (>5 kDa) were identified as the key inducers of astringent mouthfeel of red wines by means of a molecular sensory science

approach (Hufnagel and Hofmann, 2008). Flavonol glycosides, such as 3-*O*-glucosides and 3-*O*-galactosides of quercetin, syringetin and isorhamnetin, have been reported to be astringent at very low detection threshold levels. The phenolic acids in wines, especially hydroxycinnamic and benzoic acid derivatives, have been reported to be more puckering astringent, whereas flavonol glycosides were more velvety astringent (Hufnagel and Hofmann, 2008).

1.1.5 Balance

Palate balance is a critical feature influencing wine quality because it contributes to the harmony and integration of structural components. The major factors governing palate balance in dry wines are the quantity and quality of tannins, concentration of alcohol, and concentration and types of acidity. The palate balance formula is functionally analogous to the suppleness index described by Peynaud *et al*., (1980):

Suppleness index = alcohol (vol/vol) - (titratable acidity + tannin)

This formula indicates that perception of sweetness derived from alcohol, polysaccharides and sugar (when present), must be in balance with the sum of perceptions of acidity, astringency and bitterness. This relationship suggests that wines lower in acidity and/or higher in alcohol could require more tannin to be in balance. In reality, wines high in ethanol and low in titratable acidity, and without the right amounts of tannins, are often described as broad and flabby.

High Et-OH, low TA = Flabby, Broad Wine

The opposite is also possible where wines are described as harsh, even with moderate amounts of tannins, when low ethanol and high titratable acidity are the main features of the wine.

Low Et-OH, high TA = Harsh Wine

Wine is a complex matrix and its taste perception is determined by a balance among all of the sensory active compounds like acids, sugars, ethanol, and others. All these gustatory *stimuli* have been shown to affect the perception of astringency. Adding acids to wines or tannic acid solutions produced an increase in astringency (Guinard *et al.*, 1986). On the contrary, the addition of sweeteners was observed to attenuate the mouth dryness typical of the astringent sensation both of tannic acid solutions (Lyman and Green, 1990) and red wine (Ishikawa and Noble, 1995). The astringent sensation can also be altered by the presence of the most important component of wine: the ethanol (Lesschaeve and Noble 2005). As the ethanol level increased in model solution (Fontoin *et al*., 2008), a decrease in perceived astringency and in some astringent subqualities (Vidal *et al*., 2004a) was observed. In case of red wine, astringency sensation was affected by alcohol reduction (Meillon *et al*., 2009). Astringency can also be modulated by the presence of some polysaccharides, such as mannoproteins, decreasing the intensity of some astringent attributes and contributing to the fullness of model wine solutions (Vidal *et al*., 2004b).

1.2 Polyphenols–salivary proteins interactions

1.2.1 Introduction

The biological activities of tannin are related to its interaction with protein. This interaction has some influence on the functional properties of ecological and agricultural

systems (Zurker, 1983; Aerts *et al.*, 1999). The importance of tannin-protein interactions in ecological systems is illustrated by the reduced palatability of high tannin forages to herbivores (Mehansho et al., 1987). This process is believed to be due to the astringent sensation experienced when salivary proteins are precipitated by tannins and as a result lose their ability to lubricate the epithelial membranes of the mouth (Goldstein and Swain, 1963). Some of the salivary proteins have the ability to bind the tannins in vegetable derivates as red wine, tea and strawberries, which reduces the binding of tannins to the oral mucosa and their absorption from the gastrointestinal tract (Austin *et al.,* 1989) Tannins have the ability to inhibit several digestive enzymes and to precipitate other proteins. The presence of interacting proteins in saliva enables us to avoid most of the deleterious effects of tannins (Austin *et al.*, 1989). Saliva is a biological fluid with a multifunctional role that makes it interesting in terms of research and diagnostic possibilities, because of the great diversity in proteins and peptides. Saliva constituents as well tannins composition are of a great importance for establishing protein-tannin interactions. Different methods able to measure *stimuli*, in particular tannins, and protein-tannin binding were afterwards presented.

1.2.2 Human saliva

There is evidence that saliva may affect the way we perceive the taste and mouthfeel of foods in various ways (Christensen, 1985; Fisher *et al*., 1994; Spielman, 1990). The whole saliva continuously baths the oral cavity, acting as a buffering system. During wine tasting, saliva transports and dissolves the stimuli substances (Matsuo, 2000).

Although saliva is predominantly a watery fluid, it also consists of a complex mixture of proteins, ions and other organic compounds produced mostly by the salivary glands. Until now, more than 2000 different proteins and peptides have been identified in whole saliva and salivary glandular secretions (Bandhakavi *et al.,* 2009). From these, more than 90% derive from the secretion of the three pairs of "major" salivary glands (parotid, submandibular and sublingual glands). The remaining 10% derives from "minor" salivary glands and from extra-glandular sources (Humphrey and Williamson, 2001).

More than 95 percent of salivary protein is from the major salivary protein families, which include acidic and basic proline-rich proteins, amylase, high- and low-molecular-weight mucous glycoproteins, agglutinins, cystatins, histatins and statherin (Helmerhorst and Oppenheim, 2007) After protein synthesis in the salivary glands, many of these proteins undergo posttranslational modifications, which include glycosylation, acylation, deamidization, sulfation, phosphorylation and proteolysis, before they enter the mouth (Dawes, 2008).

1.2.3 Polyphenol-protein interactions

Nowadays, the interaction between proteins and proanthocyanidins is widely recognised to be a combination of hydrogen bonding and hydrophobic effects in the acidic wine matrix. Murray *et al.* (1994) used NMR to identify stacking between the phenolic rings of the proanthocyanidins and proline residues with hydrogen bonding between the hydroxyl groups on the phenolic B-ring and hydrogen acceptor sites of the peptide bond. Similar results were obtained from isothermal calorimetry (ITC) studies based on changes in entropy, reflecting the hydrophobicity and conformational changes, and on enthalpy, reflecting the consequences of hydrogen bonding (Poncet-Legrand *et al.*, 2007). The aggregation of polyphenols with salivary proteins seems to be firstly mediated by hydrophobic forces and then hydrogen bonding has been postulated to provide strong and directional bonding that stabilizes the complex. The stability of these complexes depend on the tannins dimension and on the number of free phenolic groups.

1.2.4 Molecular binding

Some proteins in human saliva chemically interact with polyphenols, leading to the formation of protein-polyphenol complexes that are able to stimulate mechanoreceptors connected with trigeminal nerve, and thus transmitting to brain the perception of astringency (Trulsson and Essick, 1997).

Given that the carbonyl function of salivary proteins are very effective hydrogen bond acceptors (Luck *et al*., 1994), it would appear that they would play a significant role in bonding to polyphenols hydroxyls (Haslam, 1974; Hagerman and Butler, 1981).

Beyond the physiological, especially the salivary protein composition (Lu and Bennick, 1998) and psychological factors (Martens, 1999) that mediate its perception, new physico-chemical quantities (binding constants, stoichiometry and atomic structure of complexes, driving forces for association, etc.) have been utilised recently to better understand the multifaceted sensation of astringency. Many techniques including circular dichroism (CD) (Jöbstl *et al*., 2004), fluorescence spectroscopy (Soares *et al*., 2007), dynamic light scattering (DLS) and nuclear magnetic resonance (NMR) (Gonçalves *et al*., 2011) have been employed to understand the formation mechanism of protein/polyphenol aggregates in solution. Generally, these studies focused on interactions between protein segment from human saliva PRPs proteins family and different procyanidins, because it represents the easiest way to simulate such a complex phenomenon. The driving factors that determine the binding between tannins and salivary proteins were identified to be the critical micelle concentration value (CMC), tannin structure preferences and tannin colloidal state (Cala *et al*., 2010). Below this value (from 1.5 to 2.9 mM as observed in wine), tannins specifically interacted with proteins through hydrophilic recognition. Depending on tannin conformation this can led to the formation of a network of interactions and then to the precipitation of the complex, or if a intramolecular staking ∏-∏ of phenolic groups is preferred the precipitation is not observed. Above this value, tannins spontaneously tend to form aggregates that at first through specific interactions bind proteins and then surrounded by the hydrophobic residues stabilize the complex by hydrophobic bonding. Both hydrophilic and hydrophobic interactions contribute to a more complex network, that determines the precipitation of salivary proteins with tannins.

1.3 Instrumental analyses for assessing astringency

1.3.1 Introduction

A method for measuring astringency remains one of the great analytical challenges in wine chemistry and practical enology. The interest into investigating on the mechanisms and interactions between polyphenols and proteins would help to identify the optimal way to simulate and evaluate what happens during red wine tasting. Quite often, the sophisticated techniques utilised, the purification of both tannin and protein fractions, the extrusion from the wine content, and the omission of matrix components during reactions all contribute to send away astringency from the reality that is to say wine polyphenols interacting salivary proteins in mouth, causing drying sensations.

Different procedures have been investigated during last decades for measuring tannins and for their exhaustive characterization, the chromatographic and spectrophotometric techniques probably offer the best methods currently available. Other ways to approach to the phenomenon of astringency, as the precipitation protein assays and the turbidimetric analysis have been developed.

Sensory analysis represents the human response as analytical tool to evaluate wine profiles. Many training and tasting sections are necessary over a long period involving a high number of tasters, then a statistical analysis provides an objective evaluation of the

sensory properties of the wine. In the case of astringency, it is very difficult to discern among tastes and brings on fatigue. A method capable of estimating tannin palatability has to be the most objective as possible and must correlate with sensory data in order to reflect the mechanism of wine tasting.

1.3.2 *Stimuli* analysis: *pro et contra*

Among *stimuli* able to elicit astringency, tannins are the main compounds responsible for this sensation. Tannins are intrinsically amphiphilic molecules with high reactivity, have a diverse range of structures, and are often found in matrices with other phenolic molecules containing similar functional groups. Besides using sophisticated equipment and analytical techniques, there is also a great interest for a relatively simple method.

A typology of measure is based on colorimetric techniques. The density of color produced by various reactions is read spectrophotometrically and compared with a standard. The major defect is that every phenolics compound produces a different color yield per unit mass in the colorimetric assay. The first method reported in literature was carried out to determine all the mixture of phenolics compounds. The oxidative technique for measuring total phenols was first described by Folin and Denis (1912). Folin-Denis reagent is an aqueous solution of sodium tungstate, phosphomolybdic acid, and phosphoric acid, which is reduced to a blue complex of tungsten and molybdenum oxides by phenolics. The reagent is added to the sample with an excess of saturated sodium carbonate solution. The color is allowed to develop for a constant time period and is read at 725 nm (Swain and Hillis, 1959) or at 760 nm (AOAC, 1965; Laurent, 1975). Because of some interferences the assay was modified into the Folin-Ciocalteau assay.

More specific color reactions exist which can be used to measure condensed tannins and their precursors. The proanthocyanidin tannins yield small amounts of anthocyanidins when treated with hot mineral acid, hence their name (Swain, 1979); anthocyanidins are colored compounds and the amount produced can be quantified spectrophotometrically. Heating the plant extract with HCl and n-butanol produces a red color which is read at 530-550 nm (Swain and Hillis, 1959; Feeny and Bostock, 1968; Cooper-Driver *et al.,* 1977; Balick *et al.*, 1978; Swain, 1979). Also vanillin dissolved in sulfuric acid (Swain and Hillis, 1959; Laurent, 1975) or in hydrochloric acid (Burns, 1963) reacts with resorcinol-type phenols to produce a rose color. The reagent is added quickly to the sample, and the color is read at 500 nm after a constant time period of 15 or 20 min.

These analytical methods currently available for determining tannin have several disadvantages. The functional group methods do not have satisfactory specificity. For example, the redox methods such as the Folin-Denis assay (Folin and Denis, 1915) are not specific for tannin, but detect any phenolic compound. On the other hand, the proanthocyanidin and vanillin assays (Bate-Smith, 1975; Price *et al*., 1978) are too selective. The hydrolyzable tannins, which are gallic acid derivatives (Haslam, 1979), do not react with acidic butanol or vanillin. Only the flavonoid-based condensed tannins (Haslam, 1979) can be detected with these reagents.

Other methods based on the acid cleavage are based on the acid-catalysed condensation reactions with benzyl mercaptan and phloroglucinol. The thiolysis and phloroglucinolysis analysed condensed tannins determining both the chain length and composition by HPLC. Proanthocyanidins become depolymerized, releasing terminal subunits as flavan-3-ol monomers and extension subunits as electrophilic flavan-3-ol intermediates. The electrophilic intermediates can be trapped by the nucleophilic reagent to generate analyzable adducts. Most of our current knowledge about general composition and structure of grape and wine tannins has been obtained by depolymerization (Lorrain *et al*., 2013).

Poor yields due to reaction product instability, reactions with nonproanthocyanidin compounds, and side reactions also contribute negatively to the utility of thiolytic methods (Matthews *et al.*, 1997). The problem with phloroglucinolysis on the other hand is that produces low yields and only a fraction of the tannin is converted to known flavan-3-ol products (Mc Rae *et al*., 2010).

1.3.3 Precipitation assays: *pro et contra*

An interesting aspect of the protein precipitation assays is that the interaction of proteins with tannins can be used to model astringency perception in humans (Bate-Smith, 1973). Protein-precipitating capacity has traditionally been measured using hide powder or gelatin. Laurent (1975) claims that the results obtained with the hide powder technique are variable, and attributes this to the long, involved procedure which may result in tannin loss, particularly when dealing with low concentrations. Bate-Smith (1973) also comments on the laboriousness of the procedure, and points out that the protein of skin is not the same as the protein of saliva, the precipitation of which accounts for the "puckery" sensation induced by tannin. The use of gelatin is discussed by Farnsworth (1966) and by Nierenstein (1934), who states that gelatin precipitates phenols other than tannins, such as hydroxyhydroquinone, gallic acid, and protocatechuic acid. Swain (1979) notes that this is also a problem when hide powder or polyvinylpyrrolidone are used in high concentrations. Bate-Smith (1973a) has introduced a spectrophotometric technique for measuring the relative astringency of tannins which he calls hemanalysis. The plant extract is mixed with dilute blood, and the optical density of the hemoglobin is measured after the tannin-hemoglobin precipitate has been removed by centrifugation.

Goldstein and Swain (1965) developed another technique for measuring relative astringency spectrophotometrically. They measured the inhibition of enzymes such as β-glucosidase by tannic acid and condensed tannins through determining the amount of residual enzyme activity in the supernatant after centrifugation of the tannin-enzyme complex, β-glucosidase acts on the glycoside substrate aesculin to produce an aglycone, aesculetin, which forms a colored chelate with aluminum chloride. The reading at 385 nm for 10 min of the rise in absorbancy gives the amount of aesculetin produced, and, therefore, the activity of the enzyme left in the supernatant, i.e., the amount of enzyme not complexed with the tannin. The level of enzyme inhibition is expressed as percentage of activity of the control.

Alternatively, Hagerman and Butler (1978) advocated the addition of bovine serum albumin (BSA) as a precipitant to tannin solutions and numerous modifications of this principle have been reported, each varying in technical requirements. An expansion of the BSA assay has been reported (Harbertson *et al.*, 2003) for use with wine, whereby the precipitation step was complemented with the bleaching effect of bisulfite. This modification allows additional quantification of small polymeric pigments (non-bleachable and non-precipitable) and large polymeric pigments (non-bleachable, but precipitable). A “gelatin index” has also been proposed (Glories 1984), whereby tannins are ranked by their propensity for precipitation of gelatin. Nevertheless, this procedure requires proanthocyanidin concentration be determined before and after precipitation with an excess of gelatin. Besides, gelatin is a heterogeneous mixture of proteins, and its composition may change among the different commercial products. This may also be an important source of variability and imprecision. Again, the variability in composition and purity of gelatin might have caused problems with the reproducibility between studies, and has led some researchers to try ovalbumin instead (Llaudy *et al.*, 2004). Another tannin assay was the methylcellulose-precipitable (MCP) tannin assay (Sarneckis *et al.*, 2008; Mercurio *et al.*, 2007). The MCP tannin assay is based on polymer-tannin interactions, resulting in an insoluble polymer-tannin complex, which precipitates and is

separated by centrifugation. It is a subtractive measure requiring the preparation of a control and treatment sample. The control sample represents the total phenolic concentration present in the matrix, whereas the treatment sample represents the phenolic concentration remaining in the supernatant solution after the tannin has precipitated. The phenolic content is monitored by measuring the absorbance at 280 nm (A280). By subtracting the A280 of the treatment sample from the A280 of the control sample, the A280 of the tannin in a solution can be determined. The A280 can be either used as an arbitrary value or converted to monomer equivalents (epicatechin equivalents, mg/L).
The use of salivary proteins has been proposed to better represent the model system. In precipitation assays, fractionated (Austin, 1989; Yan and Bennick, 1995) or whole (Sarni-Manchado *et al*., 1999; Gambuti *et al.,* 2006) human saliva has been used. Mixing whole saliva and grape polyphenols gives rise to a "soft cloudy" precipitate, which gathered after centrifugation on the bottom of the tube so that the supernatant was easily recovered without disturbing this pellet. The binding reaction was performed at 25°C, the complex formed was successively precipitated by centrifugation at 4°C in order to stop further reactions. The induced precipitation allowed to separate the proteins bound to polyphenols from whose remained in solution that not reacted with them. Both the nature of condensed tannin (Sarni-Manchado *et al.*, 1999) and salivary proteins (Gambuti *et al.*, 2006) involved in the precipitation were studied in order to highlight some solutions to the astringency problem. In both works, the SDS-PAGE electrophoresis of human saliva was carried out, Sarni-Manchado *et al.*, analyzed the supernatant and the pellet together with tannins analysis, while Gambuti *et al.*, analyzing the supernatant, revealed the proteins mainly reacting after the binding with polyphenols by comparison with the saliva control.
However, the precipitation assays also have some disadvantages. If the assays utilizes protein different from saliva, the binding reaction seems to not reproduce the physiological conditions during wine tasting, because the binding affinity of the protein is not comparable to that of salivary protein. It has been showed that the salivary protein has a higher affinity for tannin than BSA or ribulose bisphosphate carboxylase/oxygenase. In fact, in presence of a 40-fold weight excess (10-fold molar excess) of bovine serum albumin or a fivefold weight excess (equimolar) of the enzyme, the tannin preferentially bound the salivary protein. Other proteins, including dietary proteins, may not complex any tannin in the presence of the salivary tannin-binding protein (Austin *et al*., 1989).
The use of salivary proteins involves the collection of human saliva from different volunteers according to a specific protocol and it must take into account the salivary flow because it represents an important criterion to consider in order to limit the effect of individual differences in astringency perception due to subjects' saliva characteristics.

1.3.4 Nephelometry: *pro et contra*

Nephelometry is a method that allows a direct estimation of the amount of protein/tannin complexes by measuring the scattered light in solution that results from the gradual formation of a cloudy precipitate corresponding to the aggregate. The basis of this technique was proposed by Chapon (1993) on the study of the interactions between beer polyphenols and proteins to tackle the problems of colloidal stability of beer. A continuous flow method was also used to study the interactions between grape extracts and wine with BSA at different concentrations (Carvalho *et al*., 2004). Globular proteins and PRPs were used to measure a relative tannin specific activity of procyanidin oligomers from grape seeds (De Freitas and Mateus, 2001). The strongest affinity was showed by PRPs. In contrast with this studies, BSA has been considered as the most suitable model protein, besides it is structurally different from human salivary proteins. In turbidity measurement, whole human saliva (Horne *et al*., 2002) and mucin, a protein

present also in human saliva (Monteleone *et al*., 2004), were used as model proteins for astringency assessment. Based on polyphenol/mucin reactivity a micro-plate assay, based on the ability of haze particles to screen the radiation emitted by a fluorescent compound was also developed (Fia *et al*., 2009). To the extent that salivary proteins are bound by tannins in the initial steps of the development of the astringent sensations, the salivary haze development as an indicator of the protein-tannin interactions was negatively correlated with astringency. Also in this case salivary flows of donators have to be considered. The maximum of the phenolic compounds concentration used in turbidimetry measurements was 3.2 g/L for tannic acid, 2.67 (Monteleone *et al.*, 2004) and 2.9 g/L (Fia *et al*., 2009) of grape seed extracts. However, it was also demonstrated that higher concentrations of stimuli can be perceived as astringent (Gambuti *et al*., 2006).

1.3.5 Sensory analysis: *pro et contra*

Sensory analysis represents the human response to wine tasting. A sensory panel can provide information about the sensory properties of a product, but significant training is required before the panel becomes a reliable sensory instrument. Astringency is a difficult sensory attribute to evaluate, owing to particular characteristics of the sensation. It is usually estimated by tasting and is subject to a certain subjectivity. The feeling can take over 15 s to fully develop and is known to build in intensity and become increasingly difficult to clear from the mouth over repeated exposures (Guinard *et al.*, 1986; Lyman and Green, 1990), so is difficult to reduce the carryover effects. When wines or tannic solutions are evaluated by a well-trained panel using established sensory methodologies, the panel leader can expect to obtain reliable information about the intensity in perceived astringency of the samples. Screening, selection, training, and panel maintenance are exercises that help the panel attain proficiency prior to product evaluation. It is also important to discuss and familiarize with the terms associated with astringency. A vocabulary of 33 terms has been proposed by a limited combined panel of experienced tasters and winemakers to describe the mouth-feel characteristics of red wines (Gawel *et al*., 2008). Methodologies widely applicated are descriptive and rating sensory analyses. The first helps to distinguish between samples by a qualitative description of their sensory properties (Vidal *et al.*, 2004a) and the second permits to scale samples according to the intensity of the perception. However, the currently most used sensory analysis temporal methodology is time–intensity (TI). This method consists in recording one by one the intensity evolution of given attributes (Lee and Pangborn, 1986). According to Pineau *et al.* (2009), TI is interesting but since only one attribute can be evaluated at a time, it is a time-consuming method which has to be carried out with a limited number of attributes or products. Moreover, the continuous assessment of temporal changes in the perception of a single-attribute is known to induce a halodumping effect (Clark and Lawless, 1994) with a carryover from perceived attributes to the one being evaluated. To overcome these drawbacks, Pineau *et al.*, (2009) developed a new method called Temporal Dominance of Sensations which consists in identifying and rating sensations perceived as dominant until the perception ends. Before the development of this method, a similar experimental approach has been successfully used to describe temporality of sensations in wines by Pessina *et al*., (2004). It has been shown that from these data, astringency takes many seconds to develop after the basic tastes, and the duration depends on wine.

Intense training is necessary to distinguish astringency from other tastes, especially bitterness. Fatigue and loss of stimuli memory, particularly with panelists unfamiliar with astringency, may occur if an expert panel do not participate to tasting sections. Training is also expensive and time-consuming. Sensory analysis is of fundamental importance, but the association with an analytical instrument to measure astringency would avoid some of these inconvenient.

1.4 Correlation between sensory and analytical analysis

Because astringency is a major factor in wine quality, winemakers are interested in an analytical and objective method to evaluate it. No method can substitute completely sensory analysis, but a method that results a reproducible index has to correlate quite well with it. A statistically significant correlation between the sensorial and analytical methods is necessary. The gelatin index represents the almost widely analytical method for estimating astringency in red wine (Glories, 1984). Besides it gives only an approximate result (Llaudy *et al*., 2004), more recently a correlation with sensory analysis was achieved by Goldner and Zamora (2010), utilizing 29 wines judged by ten panelists on a 9-point intensity scale. A positive correlation (R^2=0.563) between gelatin index and time-intensity data was obtained only at low concentration of polyphenols. In alternative to gelatin, a method that used as precipitation agent the ovalbumin, was proposed to determine astringency (Llaudy *et al*., 2004). Ten wines were tested by ten expert enologists evaluating the astringency on a scale from 1 to 100. The method resulted more reproducible than gelatin index and a good correlation (R^2=0.7737) with sensory analysis indicate a high potentiality in assessing astringency.
Another predictive models for astringency estimation was based on phenolic compounds and color analysis of 34 wines by 12 judges on a 9-point intensity scale (Cliff *et al.,* 2002). Multiple regression generated three possible models to predict astringency from analytical values, the most simple depends on total phenolics and on copigmented anthocyanins, besides the predicted astringency plotted versus observed astringency resulted in low correlation but considered acceptable from a sensory perspective.
Monteleone *et al*. (2004) proposed a predictive model by measuring the polyphenol-mucin reactivity in which the capability of polyphenolic extracts to induce astringency can be estimated on the basis of their ability to develop turbidity in the *in vitro* assay; they found a linear relation between astringency perceived by thirty trained judges on 7-point category scale (from not astringent to extremely astringent) and the astringency mucin index for tannic acid model solutions (R^2=0.093) and grape seed extracts (R^2=0.096).
In a study of Kennedy *et al*. (2006), forty red wines were evaluated by a panel consisted of three winemakers and two enologists for the astringency intensity scored from zero to 10, with zero values being assigned when there was an absolute absence of astringency and an intensity score of 10 representing the highest imaginable astringency. The aim was to correlate astringency and tannin concentration measured by different analytical methods: absorption at 280 nm, phloroglucinolysis, gel chromatography and BSA protein precipitation. The analytical method having the strongest correlations with perceived astringency was protein precipitation (R^2 = 0.82). Because the protein precipitation method is similar to the physiological response to astringents, it could become an important in vitro tool for understanding how tannin structure modification leads to modification in astringency perception.
Generally, it was assumed that the most suitable proteins for evaluating astringency are the salivary proline-rich proteins (PRP). However, it is very difficult to obtain enough PRP as their purification is highly complicated. Nevertheless, proteins as gelatin, ovalbumin, mucin, whole human saliva have been used in different methods that would measure astringency. In order to consider a method as the most objective as possible it has to correlate instrumental with sensory data. In all cases a relationship with the human evaluation was obtained.
The great challenge is to avoid the sensory evaluation especially when many wine samples are analyzed in winery prior to a fining treatment. This is only an example of the future applications that an analytical method may offer to winemakers and enologists to

manage wine style and quality. Further researches will be focused on the possibility to create a simple and rapid method based on the SPI, able to measure astringency in winery as well in laboratory.
Under the sensory point of view, it is less important to know whether a given wine contains more epicatechin than catechin, or if the polymers are more galloylated than monomers, than the knowledge of how whole polyphenolic pattern of wine reacts with salivary proteins in mouth to generate astringency and how its characteristics change over time. This represents the real tool for help wine producers to improve wine quality.

1.5 References

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''A man should look for what is, and not for what he thinks should be.''

(A. Einstein)

Chapter Two

Aims of the thesis

The aims of the thesis were resumed as follows:

1) Development and validation of the SPI method in order to evaluate the astringency of red wines
2) The application of the SPI method to evaluate:
    a) Factors influencing astringency
        i. Stimuli
        ii. Medium components
    b) The effect of enological practices, in particular:
        i. Dealcoholisation
        ii. Wine fining
3) Characterization of Aglianico (*Vitis vinifera* L. cv) grape proanthocyanidins and evaluation of their reactivity toward salivary proteins by the SPI (Saliva Precipitation Index).

''The chemical complexity and heterogeneity of plant tannins means that they do not lend themselves to ready quantitative assessment, and this has produced a confused picture of their real significance – both evolutionary and ecological…''

(Haslam, 1988)

Chapter Three

Results and Discussion

3.1 Development and validation of the SPI (Saliva Precipitaion Index) method: Application of the SPI (Saliva Precipitation Index) to the evaluation of red wine astringency

The aim of this work was to evaluate the astringency of red wines by means of a SDS–PAGE based-method. The optimization of the in vitro assay, named SPI (Saliva Precipitation Index) that measured the reactivity of salivary proteins towards wine polyphenols, has been performed. Improvements included the choice of saliva:wine ratio, saliva typology (resting or stimulated saliva), and temperature of binding. The LOD (0.05 g/L of condensed tannin) and LOQ (0.1 g/L of condensed tannin) for the binding reaction between salivary proteins and tannins added in white wine were also determined. Fifty-seven red wines were analysed by the optimised SPI, the Folin–Ciocalteu Index, the gelatine index, the content of total tannins and the sensory quantitative evaluation of astringency. A significant correlation between the SPI and the astringency of red wines was found (R^2 = 0.969), thus indicating that this assay may be useful as estimator of astringency.

3.1.1 Introduction

Astringency is an important sensory characteristic of food and beverages containing polyphenols. This mouthfeel is mainly due to the interactions of polyphenols with salivary proteins, causing complexes formation and their further precipitation, which leads to a reduction of the lubricating properties of saliva (Breslin *et al.*, 1993). Consequently, sensations of dryness, hardness, and constriction are felt in the mouth (Lee and Lawless, 1991). Wine astringency is generally estimated by tasting, which represents an in vivo evaluation according to physiological responses. Notwithstanding, sensory analysis has some disadvantages: (i) the confusion of astringent sensation with sour and bitter tastes, (ii) the tendency of the astringency to increase on prolonged exposure, (iii) the necessity of an expert panel, (iv) the carry-over effects, (v) the influence of many factors, such as ethanol, pH and acidity on the intensity of perception (Fontoin *et al.*, 2008). In addition, astringency evaluation is subject to certain subjectivity (Valentova *et al.*, 2002). For this reason, a chemical evaluation of astringency has gained much attention during last years. Several studies focused on methods for astringency prediction, based on polyphenol–proteins interactions and the correlation with wine astringency perceived by trained jury. Since astringency is mainly considered a tactile sensation, different methods based on tannin precipitation were built up. The methods developed up

to now to estimate the chemical astringency (Llaudy *et al.*, 2004; Mercurio and Smith, 2008) utilised different proteic standards as precipitation agent. The utilisation of saliva has not yet been considered despite its contributing to taste and mouthfeel perception (Neyraud *et al.*, 2009) by transporting and dissolving the stimuli substances, and by interacting with them (Matsuo, 2000). The difficulties in measuring wine astringency using human saliva lies in reproducing the physiology involved during tasting. The perception of astringency is basically a dynamic process, that continuously changes and evolves to reach a maximum of intensity once red wine is ingested. Following the physiological course, what happens in mouth during wine tasting? When a sip of wine is made, a volume of wine is introduced in the oral cavity. According to sensory protocols, it is necessary to hold wine in the mouth for almost 8 s, during which wine constituents mix with saliva at a temperature of 37 °C. After this time, wine can be expectorated or ingested together with saliva. Astringency takes many seconds
(15 s) to develop fully (Kallithraka *et al.*, 2001) and is perceived on soft palate, gingives, lips as well as on the oral cavity (Breslin *et al.*, 1993). The chemical interactions of wine polyphenols with some salivary constituents and the subsequent precipitation reduce the lubricating properties of saliva, so sensations of dryness, hardness, and constriction are felt in mouth. In a previous work (Gambuti *et al.*, 2006), the precipitation of salivary proteins after the binding reaction with polyphenols was observed by means of the SDS–PAGE electrophoresis. The SPI (Saliva Precipitation Index) based on the precipitation of two salivary proteins (Rinaldi *et al.*, 2010), was utilised to evaluate the astringency of red wines. The role of human saliva in the development of astringency was also evaluated by others (Soares *et al.,* 2011), stating that in a competitive assay, the different proteins families have a different reactivity towards condensed tannins. However, not all physiological conditions involved during perception have been considered until now. In this study with the aim to optimise the SPI, the influence of parameters such as salivary flow, ratio saliva:wine, saliva typology, and temperature of binding have been evaluated. Phenolic composition of red wines was also investigated. Analytical data were then correlated with sensory analysis performed by a trained panel. On the basis of the importance that consumers and winemakers ascribe to wine astringency, a relationship between SPI and wine phenolic content, estimated in gallic acid equivalent, has been determined.

3.1.2 Materials and methods

3.1.2.1 Reagents

Solvents of HPLC grade, L(+)-tartaric acid were purchased from J.T. Baker (Levanchimica; Bari, Italy). Caffeine was purchased from ACEF (Piacenza, Italy). Tannic acid was purchased by Extrasynthése (Lyon, France). Condensed tannin (CT), characterised by a mean degree of polymerisation of 2.1, and by a flavanol composition of 45.3% (+)-catechin, 43.1% (-)-epicatechin and 11.6% (-)-epicatechin gallate (Fontoin *et al*., 2008), was provided by Laffort (Biotan, Bordeaux, France).

3.1.2.2 Wines and CT wine solutions

Aglianico and Pinot noir used for method optimization, and 57 red wines utilised for Pearson's correlation which included Aglianico, Merlot, Cabernet Sauvignon, Syrah and Sangiovese, were commercial products. CT wine solutions were composed of a white wine (Tavernello by Caviro, Faenza, Italy) with an alcohol content of 11% v/V, pH = 3.02, titrable acidity = 4.9 g/L of tartaric acid, fortified with 2% of ethanol, and added of CT at 0.1–1.0–2.5–3.5–5.0 g/L concentrations. CT wine solutions were utilised for SPI calibration and sensory analysis.

3.1.2.3 Phenolic analysis

Total phenolics (Folin–Ciocalteu Index, FCI) were measured according to Waterhouse (2001) and were expressed in g/L of gallic acid equivalent (GAE). Gelatin index was evaluated according to Glories methods (1984). Tannins (g/L) were evaluated according to Bate–Smith method (1954).

3.1.2.4 Sensory analysis

3.1.2.4.1 Training and selection sessions

Twenty-four subjects were recruited from the University of Naples ''Federico II'', (Corso di Laurea in Viticoltura ed Enologia, Avellino, Italy), to participate in sensory sessions. Eighteen were chosen on the basis of their sensory capabilities, interest and availability. Screening tasks included basic taste and binary mixtures identification, and intensity rating tests. Panelists were familiarised with samples and tasting procedures in ten one-hour training sessions. They had been introduced first to the theory of astringency and extensively trained to differentiate astringency from bitterness and sourness using 3.0 g/L tannic acid, 0.25 g/L caffeine and 4.0 g/L tartaric acid as examples of astringency, bitterness and sourness, respectively. Successively, they were asked to individuate the different stimuli (acid, sweet, sour and astringent) in binary mixtures first in water then in white wine. All the eighteen panelists indicated an ability to discriminate among these taste stimuli. In the following sessions, panelists were familiarised with astringency rating. They were asked to evaluate overall astringency of different concentrations (from 0.1 to 5.0 g/L) of CT tannin on a 9-point scale (named: absent, very weak, weak, weak moderate, moderate, moderate strong, strong, very strong, extremely strong) first in water and then in white wine. The different concentrations were not anchored to specific intensity categories. In each session five unknown samples (10 mL) were presented in balanced random order at room temperature (18 ± 2°C) in black tulip-shaped glasses coded with 3-digit random numbers. The assessors were instructed to pour the whole sample in their mouth, hold it for 8 s, expectorate and rate the perceived overall astringency using a 9-point scale. Judges waited for 4 min before to rinse with de-ionised water for 10 s twice, and then waited at least 30 s before the next sample. Each sample was evaluated within 5 min. Astringency was expressed as the maximum of intensity perceived. The data obtained were used for assessing the reliability and consistency of the panelists, which were considered acceptable ($P < 0.05$ for reproducibility of scores of replicate samples).

3.1.2.4.2 Absolute threshold determination

The rapid method (E 679) of the American Society for Testing and Material (ASTM) was used to evaluate the astringency threshold of CT concentration in wine. The absolute threshold was evaluated in a white wine prepared by adding different amounts of CT. The panelists each received six 3-Alternative Forced Choice (3-AFC) tests with ascending concentrations spaced by a factor of 1.8, starting from 0.02 to 0.38 g/L in wine. In each 3-AFC presentation, three samples are presented: two are controls, and one contains the substance under test. The judges have to examine each sample from left to right and select the odd wine. Randomization of the position of the different sample, within each 3-AFC presentation, was carried out for the different panelists; the option of going back to repeat the evaluation of each sample was possible within the single 3-AFC presentation but not possible among the different 3-AFC groups of wines. The best estimate threshold (BET) for each subject was evaluated as the geometric mean of the highest concentration missed and the next higher concentration. The group BET is the geometric mean of the individual ones. As shown in Fig. 1, the detection threshold was 0.12 g/L of CT.

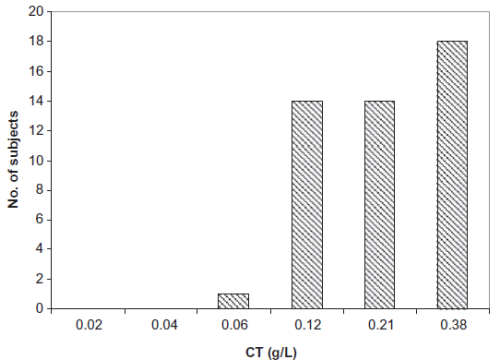

Fig. 1. Histogram of individual best estimate threshold.

3.1.2.4.3 Sensory evaluation sessions

During the tasting sessions, CT wine solutions (0.1–1.0–2.5–3.5–5.0 g/L), were evaluated for building up the calibration curve. At the beginning of each session, panelists tasted the standard reference solutions for astringency, which consisted of three CT wine solutions (very weak astringency = 0.1 g/L; moderate astringency = 2.5 g/L; extremely strong astringency = 5.0 g/L) representing the intensities of the sensation on the 9-point scale. The same was made during tasting sessions for red wines evaluation. During the tasting sessions four samples (CT wine solutions or wines) were evaluated in duplicate. In each session samples (10 mL) were presented in balanced random order at room temperature (18 ± 2 °C) in black tulip-shaped glasses coded with 3-digit random numbers. The evaluation procedure was the same as the training sessions.

3.1.2.5 SPI analysis

3.1.2.5.1 Resting saliva (RS) and stimulated saliva (SS)

Resting saliva (RS) was obtained by mixing resting saliva samples from different individuals. Stimulated saliva (SS) was obtained by chewing a paraffin piece. Saliva collection was performed between 10 to 11 a.m. Subjects were asked not to consume any food and beverage for 2 h before saliva collection. Saliva was collected from six non-smoking volunteers (three males and three females) by expectorating saliva into a pre-weighted ice-cooled tube for 5 min. The resulting mix was centrifuged at 10,000 g for 10 min to remove any insoluble material, and the supernatant was referred to as RS or SS. RS and SS flow rates of selected panelists were measured at 11 a.m and 5 p.m during the day, repeated for three days. Flow rates were determined gravimetrically and expressed as millilitre per minute (mL/min).

3.1.2.5.2 Binding assays

Interaction mixtures (150 µl final volume) contained 100 µl of saliva (RS or SS) and 50 µl of wine, which was previously filtered with Durapore filter at 0.45 µm (Millipore; Rome, Italy). Binding assays were performed in Eppendorfs maintained at 25 or 37 °C for 5 min. The mixture was then centrifuged for 10 min at 10,000 g. The analyses were performed on the resulting supernatant. Binding assays of samples were performed in quadruplicate.

3.1.2.5.3 SDS–PAGE electrophoresis

The SDS–PAGE electrophoresis of RS and SS before and after the binding assay was performed on a Bio-Rad Protean II xi Cell electrophoresis apparatus (Bio-Rad, Milano, Italy) using a PowerPac 1000 Bio-Rad power supply set at 150 V/gel for the stacking gel and 180 V/gel for the resolving gel. Samples mixed with an equal volume of 2x electrophoresis sample buffer (0.125 M Tris–HCl, 4% SDS; 20% v/V glycerol, 0.2 M

DTT, 0.02% bromophenol blue, pH 6.8) and heated at 95 °C for 4 min were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using 30% acrylamide/bisacrylamide (37.5:1) solution. The resolving gels were 14% acrylamide, stacking gels were 5% acrylamide. The gels were fixed with a mixture of ethanol, acetic acid, and deionised water (40:10:50) for 1 h. After washing in water for 5 min, the gels were stained with Coomassie Brilliant Blue R250 staining solution (Bio-Rad, Milano, Italy). The destain step was performed by incubation in the destain solution Coomassie Blue R250 (Bio-Rad, Milano, Italy). The apparent molecular weights of bands were calculated from the linear regression equation of log molecular weight against mobility, by comparison with the migration rates of Broad Range protein standards (Bio-Rad, Milano, Italy).

3.1.2.5.4 Densitometry
Densitometric tracing of gels was performed with a Bio-Rad GS800 densitometer, and electrophoretic data were analysed by Quantity One analysis software, Version 4.5 (Bio-Rad).

3.1.2.5.5 SPI (Saliva Precipitation Index)
The percentage of density reduction of two bands (at 59–54 and 15 KDa), was calculated after the binding reaction of RS with CT wine solutions (0.1–1.0–2.5–3.5–5.0 g/L). Since increasing CT concentrations in wine an increase of astringency intensity by panelists was perceived, the calibration curve (R^2 = 0.9907) was built plotting the percentage of selected bands reduction and CT concentrations (g/L) determined the SPI (Saliva Precipitation Index).

3.1.2.6 Statistical analysis
Multifactorial ANOVA with second-order interactions was used to evaluate the relationships between saliva typology and temperature with SPI. Differences of $p < 0.001$ were considered significant. As one-way ANOVA analysis, Fisher's Least Significant Differences (LSD) procedure was used to discriminate among the means of the variables. Elaborations were carried out by means of Statgraphics 5.0 Plus-PC (Manugistics, Inc.).

3.1.3 Results and discussion
The in vitro assay to evaluate astringency consists of a binding reaction between saliva and wine, subsequent centrifugation, and analysis of proteins remained in supernatant by the SDS–PAGE. The SPI (Saliva Precipitation Index) (Rinaldi *et al.*, 2010) was improved with the aim to make the in vitro assay closer to the in vivo evaluation of astringency. In this study, the bands at 59–54 and 15 KDa were chosen from the salivary pattern as representative of whole salivary proteins because the percentage of their optical density was better correlated with sensory analysis made with a trained jury on the astringency sensation. In order to optimise the method, the operative conditions of the binding reaction between saliva and wine, which is at the basis of the tactile sensation of astringency, were improved. Improvements over the existing SPI that utilises human saliva for astringency assessment include the saliva:wine ratio, saliva typology, and temperature of binding.

3.1.3.1 Parameters involved in binding reaction
3.1.3.1.1 Choice of saliva:wine (S:W) ratio
In order to choose the optimal ratio of saliva:wine, it is important to take into consideration what happens during the sensory evaluation of astringency. The in vitro assay represents a static system while the oral cavity is dynamic, in which saliva is

continuously secreted and ingested. In order to make the two systems closer, in the binding reaction wine is considered the static fluid, while saliva the dynamic one. During tasting, a volume of 10 mL of wine is generally introduced in the mouth. The volume of saliva normally present in mouth, called residual saliva, is about 0.75 mL (Lagerlof and Dawes, 1984). Beside residual saliva, a continuous flow of further saliva is secreted by the major and minor salivary glands in the oral mucosa. The salivary flow responsive to wine is 1 mL/min (Hide and Pangborn, 1978). According to sensory protocols, when wine is introduced in mouth and 15 s are necessary for astringency to develop, the volume of saliva produced within 15 s resulted to be 0.25 mL. The total salivary volume that comes in contact with wine and produced within 15 s resulted 1 mL, which is made by the sum of 0.25 mL (saliva responsive to wine during 15 s) and 0.75 mL of residual saliva. Therefore, the salivary volume par minute resulted 4 mL. In our sensory sets, the astringency evaluation of each sample, expressed as the maximum of intensity perceived, was performed within 5 min in order to avoid carry-over effects and to limit fatigue. Considering that, the in vitro binding assay between saliva and wine lasts 5 min (Sarni-Manchado *et al.*, 1999), the volume of saliva becomes 20 mL. Since 10 mL of wine is exposed to 20 mL of saliva for 5 min, the ratio saliva:wine 2:1 seems closer to reality. Starting from this ratio, the quantity of saliva was reduced progressively respect to wine. Then, other ratio saliva:wine (S:W) considered were 1:1, 1:2, 1:3, 1:4. In Fig. 2, the electrophoretic pattern of salivary proteins after the interaction of saliva and red wine at different ratio is shown.

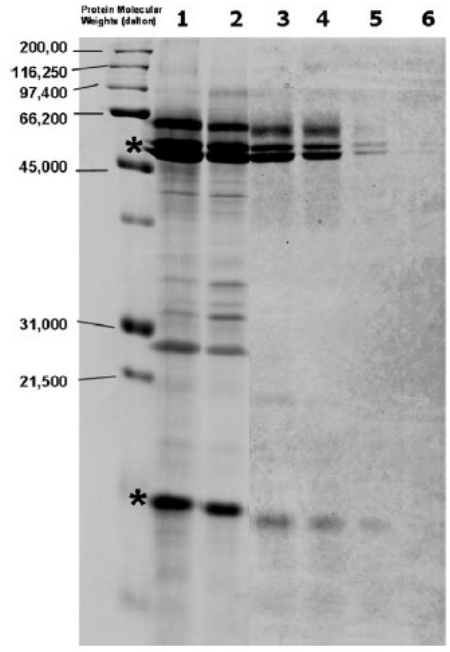

Fig. 2. The SDS–PAGE electrophoresis of human saliva (lane 1) after the binding reaction at different saliva:wine ratio (S:W), lane 2 (S:W 2:1), lane 3 (S:W 1:1), lane 4 (S:W 1:2), lane 5 (S:W 1:3), lane 6 (S:W 1:4).

The ratio saliva:wine (S:W) was 2:1, 1:1, 1:2, 1:3, 1:4 respectively in lanes 2, 3, 4, 5 and 6. As the volume of wine augmented respect to saliva (lane 1), the percentage of bands reduction due to both dilution effect and binding reaction between saliva proteins and wine polyphenols resulted of about 21% (S:W = 2:1), 43% (S:W = 1:1), 63% (S:W = 1:2), 73% (S:W = 1:3) and 100% (S:W = 1:4). Since a loss of sensitivity is recorded mainly for the band density at 15 KDa as more wine interacted with saliva, the ratio S:W

= 2:1 permits to better analyse the density of the two selected proteins (marked with an asterisk *). On the basis of what has been reported above, the ratio S:W = 2:1 resulted also to be closer to the in vivo evaluation of astringency.

3.1.3.1.2 Salivary flow

When attempting to model sensory–instrumental relationships it is particularly important to take into consideration the impact of physiological factors on perception. The typology of saliva that comprised resting (RS) and stimulated (SS) saliva, was then taken into account. Salivary flow of donators was measured because this factor can modulate the intensity of astringency induced by phenolic compounds (Fisher *et al.*, 1994). Furthermore, it is known that low responding subjects are characterised by high salivary flow rate and high responding subjects by low flow rate (Horne *et al.*, 2002). Therefore, before starting to evaluate the influence of saliva typology on the binding reaction with polyphenols, in this study, the mean flow for SS and RS of panelists has been evaluated. The flow rate values of SS and of RS were 1.66 ± 0.5 mL/min and 0.77 ± 0.33 mL/min, respectively, comparable to those determined by others (Enberg *et al.*, 2001). Moreover, the mean flow for SS was in the range of the medium flow group previously used to build up a predictive model of the astringency sensation (Condelli *et al.*, 2006). These data confirm that the salivary flow represents an important criterion to take in consideration in order to limit the effect of individual differences in astringency perception due to subjects' saliva characteristics.

3.1.3.1.3 Saliva typology and binding temperature

Once saliva:wine ratio has been chosen and the salivary flow of panelists has been evaluated, in order to give an overview on the optimization of the method, the physiological conditions of the binding between saliva and wine were tested. They included the saliva typology and the temperature of binding. Saliva that continuously baths the overall buccal cavity, is secreted by different mechanisms (various reflexes and spontaneous secretion) producing resting (RS) and stimulated (SS) saliva. For the in vitro assay the binding reaction between RS or SS and wine was tested at two temperatures 25 °C (as reported by Sarni-Manchado *et al.,* 1999) and 37 °C (physiological temperature). With the aim to apply this method to different red wines the effect of these parameters was evaluated on two red wines extremely different. The interaction was performed with Aglianico and Pinot noir wines because of their different polyphenol content. Aglianico represents a tannin-rich wine (HTW) instead Pinot noir is known for its low tannin content (LTW). In fact, tannin concentration measured with the Bate-Smith method, was of 4.96 ± 0.07 and 2.08 ± 0.10 g/L for HTW and LTW, respectively. As shown in Table 1, the values of SPI for RS after the interaction with HTW were of about 5 and 4 (expressed as g/L of CT) at 25 °C and 37 °C, respectively. As regard SS, the SPI was almost the same at the two temperatures considered.

Table 1
The SPI values (expressed as g/L of CT) determined for HTW (High Tannin Wine) and LTW (Low Tannin Wine), utilising different typology of saliva (RS and SS) at two temperatures of binding (25 and 37 °C).

Wines	25 °C	37 °C
HTW		
RS	5.14 ± 0.14	3.95 ± 0.30
SS	3.50 ± 0.10	3.89 ± 0.34
LTW		
RS	3.86 ± 0.10	2.03 ± 0.44
SS	2.32 ± 0.07	2.17 ± 0.19

HTW (High Tannin Wine).
LTW (Low Tannin Wine).
RS (resting saliva).
SS (stimulated saliva).

The SPI for LTW was of about 4 and 2 g/L of CT, after the binding reaction of RS at 25 and 37 °C. As for the HTW, the SPI measured for SS was the same at the two temperatures. Anyway, the values of SPI for HTW were greater than LTW, being in agreement with their tannin content. A multifactorial analysisof variance (ANOVA) was carried out to assess the influence of saliva typology (RS and SS) and temperature (25 and 37 °C) on the precipitation of salivary proteins, as well as the interactions between these factors. The F ratio and p values were obtained from the statistical analysis for the SPI. The F ratio represents the quotient between variability due to the effect considered and the residual variance. A higher value of F ratio means a more marked effect of that factor on a variable. As regard the wine with a high tannin content (HTW), saliva typology showed a greater effect (F ratio = 39.52; p-value = 0.0002) than temperature (F ratio = 8.26; p-value = 0.0207) on the SPI. While no significant differences at 99% for the temperature of binding at 25 and 37 °C were observed, it seems that the RS amplified the differences in SPI, respect to SS. The same trend for saliva typology was observed for the low tannin wine (LTW). Based on literature, prominent differences between stimulated and resting salivary secretions are seen in their protein composition, flow rate and viscosity. Whole saliva is a composite of secretions produced by the parotid, submandibular/sublingual and minor salivary glands. Under unstimulated conditions, the parotid glands contribute approx. 25% of whole saliva, the submanadibular/sublingual glands about 67% and the minor mucous glands about 8% (Schneyer, 1956; Dawes and Wood, 1973). When stimulated, parotid saliva may constitute up to 49% of whole saliva (Shannon, 1962). Parotid flow increases dramatically during stimulation, and its main role may be to produce copious, highly buffered, fluid to protect against extrinsic insult (for instance, acid).

Moreover, many proteins were secreted by parotid glands under stimulation. A high proportion of human parotid salivary proteins is proline-rich proteins (PRPs) (Bennick, 2002). Basic proline-rich proteins, in particular, have been found to interact strongly with polyphenols (Lu and Bennick, 1998). A more pronounced production of PRPs by parotid glands in stimulated saliva was observed in a SDS–PAGE electrophoresis by Schwartz *et al.*, (1995), as the abundance of deep pink-violet staining PRP bands was higher than in saliva produced by other glands. As SPI is calculated from the percentage of density reduction of two bands as representative of the whole saliva, in SS the interaction of these proteins with wine polyphenols was reduced because PRPs (present at a higher extent in this typology of saliva) competed for the binding reaction. Then, the SPI for SS is less sensible to polyphenolic variation in wine. The enhanced production of PRPs inducted by stimulation may determine a same level of interaction with tannins for HTW and LTW. Another difference in composition is that parafilm-stimulated saliva contained more of the high-density mucin species then did unstimulated saliva (Veerman *et al.*, 1992). As

mucin is responsible for saliva viscosity it is likely that the viscosity of SS is higher with respect to RS. Thus, the high viscosity of the biological fluid of SS respect to RS may determine a minor sensitivity of the saliva as analytical tool. In addition, when salivation is stimulated, the concentration of bicarbonate is higher than in resting condition; this may determine a higher buffering capacity of the oral fluid. Under stimulated conditions, the role of bicarbonate as a major buffering component will increase, as it will be available in high concentrations (Aps and Martens, 2005). Therefore, this is probably another reason by which SS showed a similar binding affinity with polyphenols depending on the typology of wine. In the case of low tannin wine (LTW), the influence of the binding temperature (F ratio = 47.42; p-value = 0.0013) was more pronounced than saliva (F ratio = 23.16; p-value = 0.0001) on the SPI, respect to HTW. The interaction between saliva typology and temperature was for both wines significant at 99% level in the same way (p = 0.0004). It seems that when wine is characterised by low tannin content, the interactions between saliva and wine were more promoted at higher temperature. This finding can be explained considering that when the affinity constant of the reaction increases with temperature, hydrophobic interactions were mainly involved (Oh *et al.*, 1980). Since the physiological temperature in the oral cavity is 37 °C, this parameter was chosen as the temperature of binding for the in vitro assay. Based on our results the choice of saliva typology is also important. Resting or unstimulated saliva, which refers to the saliva secreted in the absence of apparent sensory stimuli related to eating, was utilised as proteic model. Resting saliva baths the overall buccal cavity for more than 90% of the day (Matsuo, 2000) and respect to stimulated saliva is less subject to variability. The salivary flow rate, and consequently the composition of stimulated saliva, in fact, is influenced by the time of collection (Dawes, 1975), nature and duration of stimulus (Dawes, 1969), emotional state (Bolwig & Rafaelsen, 1972) and gender (Heintze, Birkhed, & Bjørn, 1983). Therefore, the operative conditions chosen for the in vitro assay are: resting saliva (RS) interacting with wine in a ratio 2:1 (S:W) during the binding reaction set at a temperature of 37 °C.

3.1.3.2. Method validation

Once the operating conditions for the binding reaction between RS and wine have been optimised, the repeatability of the in vitro assay was tested by carrying out twenty-two replications for reaction mixtures containing (i) saliva and white wine added with 1 g/L of tannic acid and (ii) saliva and Merlot red wine. The obtained SPIs were 1.75 ± 0.14 and 4.30 ± 0.16 g/L of CT, respectively. The RSD (relative standard deviation) for SPI were lower than 10% both for white wine supplemented with tannic acid (8.1%) and red wine (3.8%), indicating that SPI can be considered a reliable index for the evaluation of the reactions between salivary proteins and polyphenols in wine. The limit of detection LOD was estimated by the densitometric analysis of selected proteins after the binding reaction between RS and increasing concentrations of CT in white wine. The percentage of proteins reduction was determined after the addition of CT at 0.01, 0.025, 0.05 and 0.1 g/L to control wine. The LOD was calculated as the concentration at which the SPI did not differ from the white wine not added with tannin. The LOD was 0.05 g/L of CT. The limit of quantification (LOQ) sets as the concentration of CT in white wine above which the SPI became significant at 99%. The LOQ was 0.1 g/L of CT. This value was close to the absolute threshold concentration from which the trained panel became to perceive the astringency of CT wine solutions (0.12 g/L, in material and method section). The calibration curve obtained from the percentage of selected bands reduction and increasing CT concentrations (g/L) was determined. The equation of the calibration curve is $y = 19.026x + 15.858$, where x represents the CT concentration and y the SPI value. The coefficient of determination for the SPI was $R^2 = 0.9907$.

3.1.3.3 Correlation between SPI and astringency intensity

The SPI was determined for 57 different red wines. Aglianico, Merlot, Cabernet Sauvignon, Syrah and Sangiovese were included. Wines were evaluated by a trained panel for the astringency sensation. Phenolic analyses as FCI (Folin Ciocalteu Index), tannins (g/L) and gelatin index (GI) were also conducted to establish a relationship with astringency. In Table 2, the minimum and the maximum values for each analysis are shown. The tannin content of wines ranged from 1.67 to 5.55 g /L of tannins, total phenolics from 1.47 to 7.57 g/L of gallic acid equivalent, gelatine index from 24.40 to 77.98, and SPI from 2.12 to 10.18 g/L of CT. In order to assess a relationship between sensory and analytical data Pearson's correlation was carried out.

Table 2
FCI (gallic acid equivalent), gelatin index (GI), tannins (g/L) and SPI (g/L of CT) values of 57 red wines and the Pearson's correlation coefficients among sensory analysis (astringency intensity) and analytical data provided by the different analyses.

Analysis	Minimum value	Maximum value	Pearson's correlation between analysis and astringency intensity
FCI	1.47	7.57	0.648
GI	24.40	77.98	0.552
Tannins	1.67	5.55	0.800
SPI	2.12	10.18	0.969

As shown in Table 2, positive correlations were obtained for all analyses, being the gelatine index the less sensible (0.552). In a recent work (Goldner and Zamora, 2010), it was demonstrated that this method was a good estimator of astringency when polyphenols level was low, but when the concentration was higher than 5.20 g/L of gallic acid equivalent, the relationship was very low. In this study, among the 57 red wines, the analyses were carried out also on Aglianico wines characterised by high values of phenolics and tannins (maximum value for FCI = 7.57 g/L gallic acid equivalent, and for tannins = 5.55 g/L). The choice of analysing these wines was made with aim to represent a worldwide range of astringency including HTWs, as can be Aglianico or Tannat wines. As regard astringency and the FCI, the coefficient of correlation was 0.648, meaning that the phenolic content of wines is not a requisite for the potential astringency. In fact, the Folin Ciocalteu Index that measures the sample reducing capacity, has the disadvantage of an overestimation of the total polyphenolic content (Escarpa and Gonzalez, 2001). In addition, among phenols there is no a distinction from the reactive phenolics (Singleton and Rossi, 1965). The tannins measured with butanol-chloryde assay gives a better correlation with the astringency intensity (0.800), even if this method does not provide any information on the structure of proanthocyanidins. This sensation, in fact, is dependent not only on grape proanthocyanidin amount but also on structure (Chira *et al.*, 2009). In particular, astringency depends on proanthocyanidin mDP and the proportion of galloylation (Vidal *et al*., 2003). Although the SPI lacks of this information, it furnishes the reactivity of wine tannins towards salivary proteins. As shown in Table 2, a significant Pearson's correlation between the in vitro assay response and the sensory perception was obtained (R^2 = 0.969). In Fig. 3, the correlation between SPI and astringency intensity was shown (y = 0.8324x + 0.4721).

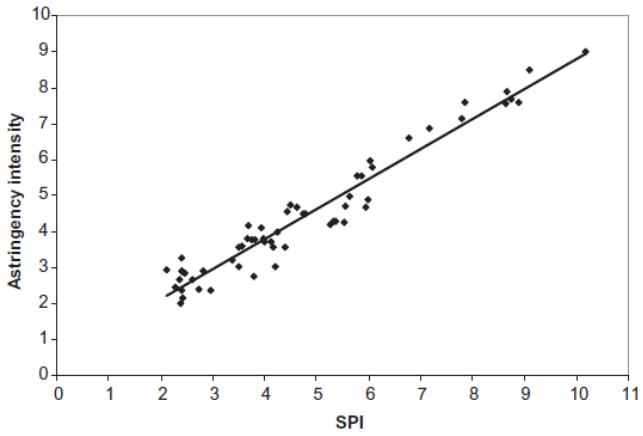

Fig. 3. The correlation between the SPI and astringency intensity.

The improved SPI method resulted a good predictor for red wine astringency. Further studies will focus on the structure characterization of grape and wine tannins reactive to salivary proteins in order to better correlate the different proanthocyanidins fractions with SPI and to determine the molecular basis of binding between tannins and each protein in salivary pattern. A relationship between SPI and gallic acid equivalent (g/L) was then obtained in order to universally quantify this index (R^2 = 0.9728). The correlation line (y = 1.0899x - 0.2128) permits to express the reactivity of salivary proteins towards wine polyphenols, and consequently the potential astringency of wines by an easy criterion of evaluation.

3.1.4 Conclusions

In order to make the SPI method more similar to the physiological response to astringents, improvements of the in vitro assay were made. The saliva:wine ratio, the choice of saliva typology and the temperature of binding were optimised as factors affecting the interactions between salivary proteins and wine polyphenols. Given the difficulties and the expense of conducting controlled sensory evaluation, the salivary proteins precipitation method (SPI) seems to be a useful in vitro tool for astringency assessment since a good correlation with this perception was obtained. The ability to quantify the astringency of red wine as gallic acid equivalent could potentially represent an easy way to express the reactivity of wine polyphenols towards salivary proteins for astringency evaluation. In addition, this study may provide a link between structural studies of binding reactions between tannins and salivary proteins and sensory perception.

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3.2 Factors influencing astringency

3.2.1 Evaluation of the astringency of commercial tannins by means of the SDS–PAGE-based method

The astringency of wines enriched with commercial tannins (CTs) was evaluated by a method based on the SDS–PAGE electrophoresis of salivary proteins after the reaction of saliva with wine. Nineteen CTs tested in synthetic wine at the same pH (3.6) and concentration (1 g/l) gave different values of saliva precipitation index (SPI). The effect of CTs addition was investigated in four wines. Results showed that the wine matrix influenced the astringent capacity of CTs and that became less pronounced as wine polyphenolic complexity increased. For some types of wine, astringency was not affected, indicating that the effect of CTs utilisation is not easily predictable by classical methods. The ability to objectively evaluate the astringency provided by CTs with the SDS–PAGE-based method would supply producers and winemakers with a useful tool to manage the processing conditions and thus to improve the quality of wine.

3.2.1.1 Introduction

The gustative balance is fundamental in defining the quality of a red wine that is determined by a fine equilibrium between three components: sweetness, acidity and astringency. Harsh wines, in which the gustative balance is shifted toward the tannic component, are usually characterised by an excess of polyphenols and are described as astringent (Gawel, 1998). In addition to polyphenols, the grape acids also contribute to wine astringency (Fischer and Noble, 1994; Guinard *et al.*, 1986). Astringency is not a taste but a mouthfeel (American Society for the Testing of Materials, 1989) perceived in the oral cavity after the ingestion of red wine as a drying, puckering, and roughing of the epithelium (Lee and Lawless, 1991). This tactile sensation derives from the interaction of salivary proteins with tannins, from their complexation,and from the subsequent precipitationin the oral cavity (Joslyn and Goldstein, 1964). Wine tannins may be categorised as condensed and hydrolyzable tannins derived from grape and wood cooperage, respectively. The former are composed of flavan-3-ol units with various degrees of substitution and polymerisation, that constitute the largest group of proanthocyanidins (Haslam and Lilley, 1988). The latter arecomposedof gallic acidand its dimer ellagic acid esterified with sugar molecules (Haslam and Cai, 1994). They have the peculiarity of binding proteins according to their structural features. In general, increasing the mean molecular mass of grape procyanidins increases their ability to precipitate proteins (Bate-Smith, 1954), as well as their perceived astringency (Vidal *et al*., 2003), up to a given degree of polymerisation. The capacity of binding protein is also enhanced by the galloylation of flavan-3-ols (Poncet-Legrand *et al.*, 2006) and by the increasing number of galloyl ester groups on the galloyl-D-glucose core (Charlton *et al.*, 2002). When the protein binding is enhanced, the perception of astringency increases (Vidal *et al.*, 2003). Therefore, the large polymeric tannins from grape skin and the more galloylated tannins from seeds extracted during the fermentation-maceration process (Aron and Kennedy, 2007) are the major grape-derived contributors to wine astringency. In addition, some gallotannins and ellagitannins, such as those released by oak barrels, can also contribute to wine astringency (Glabasnia and Hofman, 2006). At the same time, during oak ageing, some processes, such as the augmentation of the proanthocyanidin degree of polymerization and the formation of anthocyanin-flavanol adducts, occur in wine, leading to a decrease in astringency (Vivas and Glories, 1996). Currently, a common practice is the utilisation of commercial tannins (CTs) both in powders and chips, as a substitute for oak barrels, to improve colour stability and taste. A large variety of CTs from different origins is available on the market. It is of fundamental importance

to understand the effect of CTs addition on wine astringency, in order to not compromise overall wine quality. Until now the astringency of isogenous tannins has been evaluated for a few types of CTs in alcoholic solution (Vivas *et al.*, 2002) and for different purified ellagitannins in aqueous solutions (Glabasnia and Hofman, 2006; Schwarz and Hofmann, 2008) only by sensory analysis. However, the sensory evaluation of astringency is often wrongly associated with bitterness (Peleg *et al.*, 1999), and thus it is necessary to accurately train the panel; this is time-consuming and expensive. The effect on the astringency from the addition of different CTs in wine is not easy to predict because polyphenols are highly reactive and undergo several chemical transformations with other wine components inducing chemical and sensorial modifications (Cheynier *et al.*, 2006; Vidal *et al.*, 2004). In a previous work (Gambuti *et al.*, 2006) an objective evaluation of the astringency of seeds and skins by means of SDS–PAGE electrophoresis was performed. The evaluation was carried out in a reaction system which simulates what happens in the mouth during tasting. Because some salivary proteins in the electrophoretic pattern were significantly reduced after the binding reaction with grape polyphenol extracts, they were used to obtain an astringency calibration curve. Until now, this method was not applied to the analysis of wine astringency. The ability to objectively evaluate the astringency provided by tannins in wine might supply producers and winemakers with a useful tool to manage the processing conditions and thus to improve the quality of wine. In this work, in order to evaluate the astringency elicited by CTs, an SDS–PAGE-based method was applied. The relationship between CTs and the sensory perception of astringency was studied by a saliva precipitation index (SPI) measured both in model solution and wines.

3.2.1.2 Materials and methods

3.2.1.2.1 Reagents

All solvents and acids used were of HPLC grade and were purchased from J.T. Baker (Levanchimica; Bari, Italy).

3.2.1.2.2 Synthetic wine (SW)

The synthetic wine was composed of ethanol (12%) and tartaric acid (4 g/l). A pH of 3.6 was reached by adding a solution of NaOH (1 N). The synthetic wine was filtered under vacuum with sterile filters of 0.45 lm (Millipore; Rome, Italy).

3.2.1.2.3 Wines (W)

Aglianico and Merlot wines were derived from 100% Aglianico and Merlot grapes, respectively. White wine and Pinot noir wine were commercial products. After the addition of CTs, wines were stored for 72 h before measurement. Prior to the binding reaction, wines were diluted with synthetic wine in a 1:1 ratio.

3.2.1.2.4 Tannin solutions (TS)

All commercial tannins (CTs) were provided by Laffort (Bordeaux, France), except for the tannin (TA), composed of tannic acid purchased by Extrasynthese (Lyon, France). The list of tannins used in the experiments, the origin and their chemical nature is reported in Table 1. Tannin solutions were prepared in distilled water in a ratio 1:10 (tannin:water), then they were dissolved in synthetic wine at different concentrations and pH was adjusted to 3.6 when necessary with NaOH 0.1 N. After the addition of CTs to synthetic wine, 72 h passed before the measurement.

Table 1
The origin of CTs, the identity codes and the chemical class.

Origin of oenological tannins	Code	Condensed tannins	Ellagitannins	Gallotannins
Wood	TA			X
Grape	TR5	X		
Grape	TR1	X		
Grape	TR4	X		
Wood	TVS	X	X	
Wood	TV		X	
Wood	TCH1		X	
Wood	TQ		X	
Wood	TCH2		X	
Grape	TC2	X		
Grape	TR7	X		
Grape	TB	X		
Grape	TR3	X		
Grape	TR2	X		
Wood	TG			X
Wood	TCO		X	X
Grape	TR6	X		
Wood	TP		X	
Grape	TC1	X		

3.2.1.2.5 Human saliva (HS)
Human saliva (HS) was obtained by mixing resting whole mouth saliva samples from different individuals. Saliva was spontaneously collected from six non-smoking volunteers (three males and three females) by expectorating saliva into an ice-cooled tube. The resulting mix was centrifuged at 10,000g for 10 min to remove any insoluble material, and the supernatant was referred to as human saliva (HS).

3.2.1.2.6 Binding assay
Interaction mixtures (150 µl final volume) contained 100 µl of saliva (HS) and 50 µl of TS or wine. Binding assays were performed in Eppendorfs maintained at 25 °C for 5 min. The mixture was then centrifuged for 10 min at 10,000g. The analyses were performed on the resulting supernatant. Binding assays were performed in triplicate.

3.2.1.2.7 SDS–PAGE electrophoresis
The SDS–PAGE electrophoresis was performed in duplicate as reported by Gambuti *et al.* (2006) with some modifications.

3.2.1.2.8 Densitometry
Densitometric tracing of gels was performed with a Bio-Rad GS800 densitometer, and electrophoretic data were analysed by Quantity One analysis software, Version 4.5 (Bio-Rad).

3.2.1.2.9 Spectrophotometric analysis
Total phenolics (Folin–Ciocalteau Index, FCI) were measured according to Waterhouse (2001) and were expressed in g/l of gallic acid equivalent (GAE).

3.2.1.2.10 Sensorial analysis
The sensory analysis of TA samples at different concentrations (0, 2, 4, 6 and 10 g/l) was conducted as described in Gambuti *et al.*, (2006).

3.2.1.2.11 SPI calculation
The perceived astringency of TA samples at different concentrations (0–10 g/l), expressed as mean rank sum, was correlated with the percentage of band proteic reduction of the salivary proteins progressively involved. The four proteins selected for the evaluation of

astringency corresponded to the following peaks reported in Fig. 1: Peak 2 (with an apparent molecular weight of 94 kDa), Peak 3 (65 kDa), Peak 4 (54–59 kDa), and Peak 11 (15 kDa).

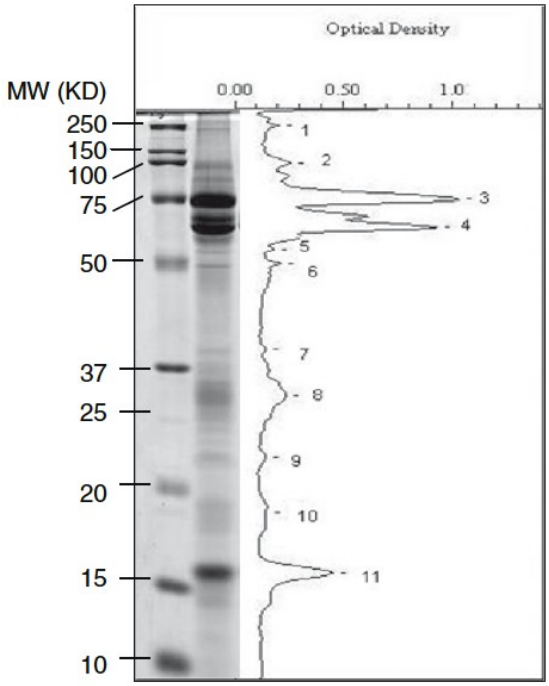

Fig. 1. The SDS–PAGE of human saliva (HS) and relative densitometric profile.

The best correlation with sensory analysis was obtained considering the mean sum of the percentages of density reduction of proteins corresponding to peaks 4 and 11 ($R^2 = 0.93$). In order to obtain a calibration curve to evaluate the astringency capacity of wine, the values of the percentage of density reduction of the two bands were plotted against the TA concentration ranging from 0 to 10 g/l. A regression line was obtained ($R^2 = 0.9685$) and used to determinemthe SPI.

3.2.1.2.12 Statistical analysis

All the data are expressed as the arithmetic average ± standard deviation of two or three replicates. Analysis of variance and Tukey's test were used to interpret differences in means, if any, at the 95% confidence level.

3.2.1.3 Results and discussion

3.2.1.3.1 Comparison among CTs

In these experiments CTs from different sources were used. The astringency of these tannic preparations at a concentration of 1 g/l in synthetic wine (ethanol 12%, tartaric acid 4 g/l, pH 3.6) was investigated using the saliva precipitation index (SPI) obtained by means of the SDS–PAGE-based method. The Folin–Ciocalteau index (FCI), as an indicator of the total phenolics of the CTs, was also measured (Fig. 2). Data reported in Fig. 2 clearly show that the content in total phenols estimated by the Folin–Ciocalteau index does not give an estimation of the potential reactivity of tannins toward salivary proteins.

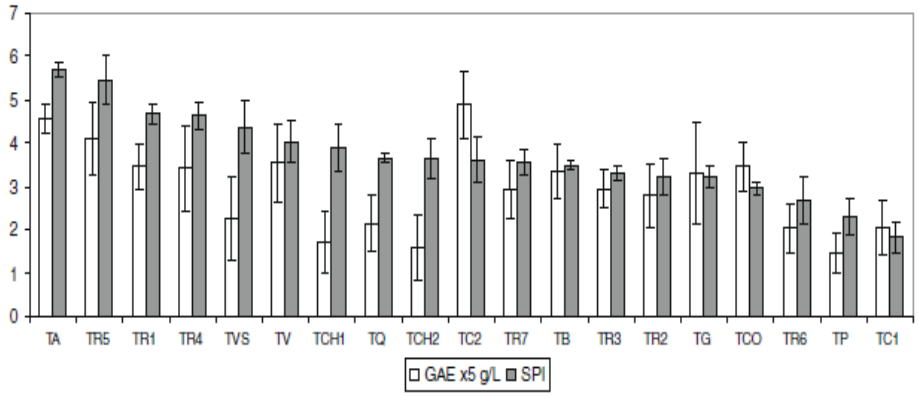

Fig. 2. The saliva precipitation index (SPI) and the Folin–Ciocalteau index, multiplied × 5 and expressed as g/l of gallic acid equivalent (GAE × 5) of the 19 commercial tannins at 1 g/l in synthetic wine. Tannins showing the same letter are not significantly different ($p < 0.05$).

Although being very similar in the total phenolics, tannins showed a different astringent capacity expressed by the SPI. As reported by Vivas *et al.*, (2002), the majority of CTs are rich in polyphenols but not necessarily in reactive tannins that represent the active material of the preparations. Since the commercial preparations of tannins are rich in other materials that vary depending on the extraction and manufacturing processes, the SPI may supply interesting information on the astringent capacity of tannins in synthetic wine, this being probably elicited by the active tannic fraction. The most astringent tannin is TA, a tannic acid which is also rich in total phenols (Fig. 2). This is in agreement with previous research that shows that tannic acid (hydrolyzable tannin containing eight galloyl groups) has an unusually high binding affinity for proteins (Hofmann *et al.*, 2006). This behaviour is due to the fact that each galloyl group provides three hydroxyl groups and a benzene ring that can establish hydrogen and hydrophobic bonds, respectively, thus increasing the binding affinity to salivary proteins as earlier observed for the binding affinity to BSA (Soares *et al.*, 2007). An interesting tannin with a low astringent capacity is TC1, composed of catechin that shows a relative low affinity for proteins. In a previous study the catechin was perceived as more bitter than astringent (Peleg *et al.*, 1999).

3.2.1.3.2 Effect of tannin concentration in model solution

The comparison of the electrophoretic pattern of salivary proteins after the reaction with four CTs: TR4, TB, TG and TP at increasing concentrations (Fig. 3), shows that the decrease of band density with concentration for condensed tannin (TR4) is more evident than for hydrolyzable tannins (TP).

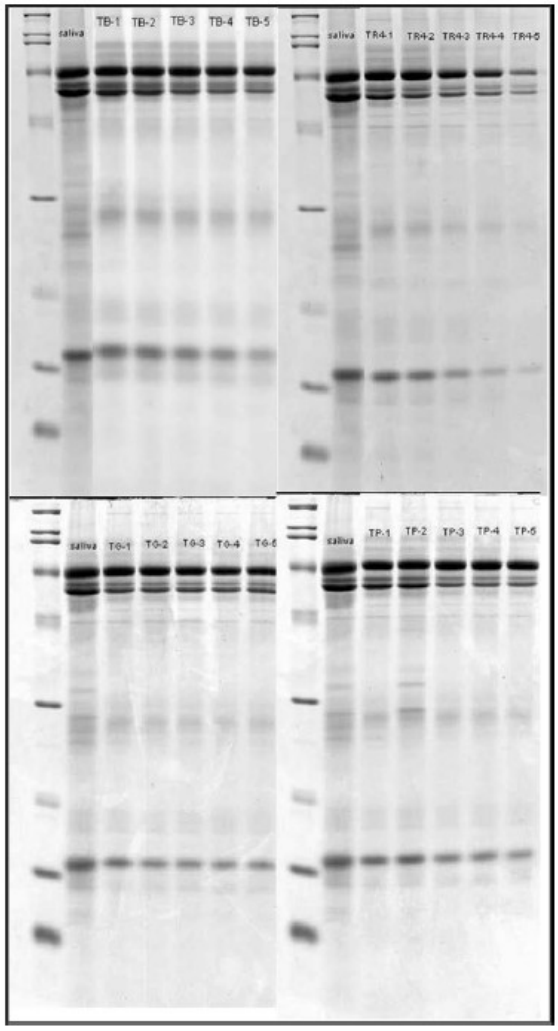

Fig. 3. The SDS–PAGE electrophoresis of salivary proteins after reaction with condensed tannins (TR4–TB), and ellagitannins (TP–TG) at different concentrations (1, 2, 3, 4 and 5 correspond to 0.25, 0.5, 1.0, 1.5 and 2.0 g/l) in synthetic wine.

The SPI of synthetic wine after the addition of increasing concentrations (0.25–2.0 g/l) of two condensed tannins (TR4 and TB) and two hydrolyzable tannins (TP and TG) was reported in Fig. 4. At low concentration there are no significant differences among tannins, showing that at 0.25 g/l tannins had a comparable reactivity toward salivary proteins. At high concentration (2 g/l) no differences in the SPI were observed indicating that the protein binding sites were equally saturated by tannins.

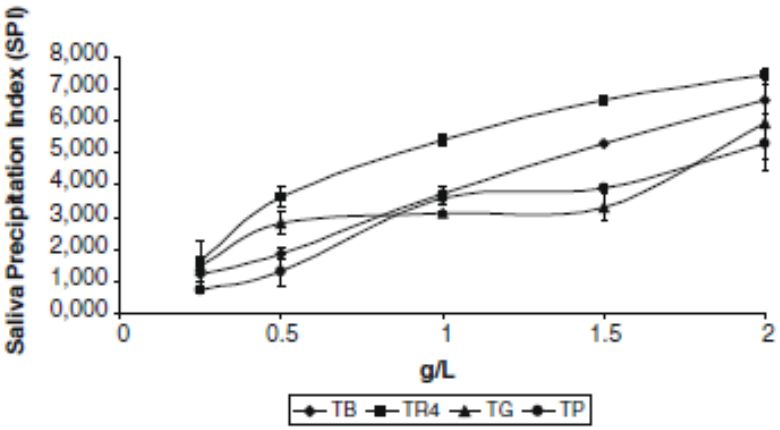

Fig. 4. Trends in the astringency index of TB, TR4 (condensed tannins) and TG, TP (hydrolyzable tannins) at increasing concentrations (0.25, 1.5, 1, 1.5 and 2 g/l) in synthetic wine.

The SPI of TR4 and TB increases with concentration (Fig. 4), as expected by the fact that the ability to precipitate salivary proteins was enhanced by concentration (Sarni-Manchado *et al.*, 1999). This result is in agreement with a previous study which showed that as the concentration of proanthocyanidins extracted from seed grapes increased, the astringency increased proportionally (Ricardo-da-Silva *et al.*, 1991). A different trend was observed for the hydrolyzable tannins TP and TG showing that the dependence on concentration was less pronounced. In this case, this phenomenon could be associated with the different ability of these tannins to form insoluble compounds with the salivary proteins (Y and S5) used in the SDS–PAGE assay. According to Hagerman *et al.*, (1998) the aggregation between polyphenols and proteins depends on the nature of the tannin–protein interactions that differ according to the polyphenolic nature of the molecules involved. In the case of condensed tannins, the interaction mechanism mainly involves the formation of hydrogen bonds, while the hydrophobic interactions seem to be responsible for the precipitation of hydrolyzable tannins. A three-stage interaction mechanism for the binding between polyphenols and peptides from salivary proteins was proposed by Charlton *et al.*, (2002). The first stage deals with the formation of a soluble complex through weak bonding, making the process reversible; in the second stage the interaction with an analogue complex caused an augmentation of the molecular mass, then the complex became insoluble. In the third stage a further aggregation with insoluble complexes led to a phase separation. In 1998, Lu and Bennick, in evaluating the stability of insoluble complexes between a representative salivary protein and condensed tannins or hydrolysable tannins, found that the insoluble complexes formed with hydrolyzable tannins tended to be more soluble than the majority of complexes with condensed tannins that instead remained insoluble.

3.2.1.3.3. The effect of wine matrix

In a second experiment, in order to evaluate the effect of the matrix on astringency, a quantity (0.3 g/l) recommended by the producers of the condensed tannin TB was added to different types of wine: a white wine, a Pinot noir, and an Aglianico wine. The SPI was measured before and after the treatment as shown in Fig. 5.

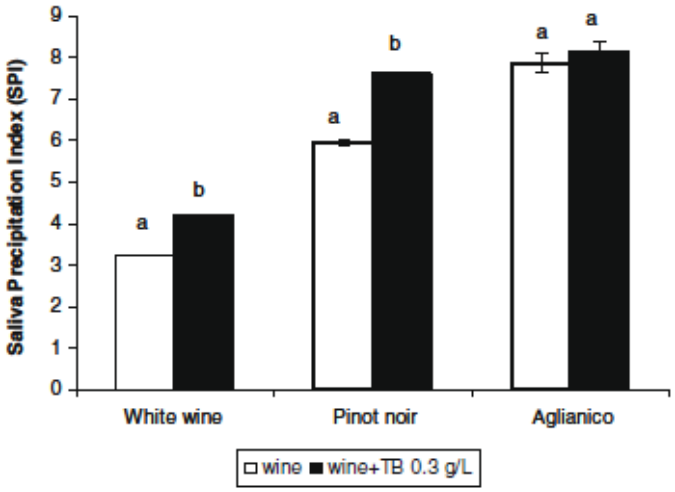

Fig. 5. The saliva precipitation index (SPI) of a white wine, a Pinot noir, and of an Aglianico wine before and after the addition of TB tannin at 0.3 g/l. Data showing the same letter are not significantly different ($p < 0.05$).

The two red wines were chosen because they differ greatly as to the content of total phenols and tannins, being higher in Aglianico wine than Pinot noir (Mattini *et al.*, 2002). The addition of the TB tannin at a concentration of 0.3 g/l enhanced the astringency of white wine and Pinot noir wine. The overall astringency of Aglianico wine was not compromised by the recommended quantity of TB. Two reasons may explain the behaviour observed: (i) the presence in wine of grape-derived tannins that have saturated the binding sites of salivary proteins, and (ii) the interaction of isogenous tannins with wine polyphenols, resulting in a loss of affinity of isogenous tannins with salivary proteins. It is interesting to note that the behaviour of white wine and Pinot noir is similar, while a great difference was observed between the two red wines. Therefore, the nature of both isogenous tannins and polyphenols of red wine is a determining factor on the effect of the addition of tannins. For these reasons, in the third experiment, three CTs (TP, TG, and TR4) were added in Pinot noir, Merlot, and Aglianico wines at 1 g/l. In Table 2 the percentage of increase of the SPI in wine after the tannin addition is shown. TR4 caused in Pinot noir an increase of the astringency of 37%, in Merlot wine of 28%, and no modification of the astringency observed in Aglianico wine.

Table 2
The percentage of increase in the SPI in wine after the addition of CTs (TR4–TP–TG) at 1 g/l in Pinot noir, Merlot, and Aglianico wines.

Tannin at 1 g/l	Percentage increase in SPI (%)		
	Pinot noir	Merlot	Aglianico
TR4 (condensed tannin)	37	28	0
TP (ellagitannin)	23	11	0
TG (gallotannin)	33	27	23

The addition of TP caused an increase of the SPI equal to 23% in Pinot noir, to 11% in Merlot and 0% in Aglianico wine. Therefore, the treatment of Aglianico with the CTs TR4 and TP does not affect the wine astringency. This result is of technological importance because these tannins can be utilised on Aglianico wine without the risk of producing harsh wine. The addition of the tannin TG resulted in an increase of the percentage of the SPI of 33% in Pinot noir, of 27% in Merlot, and of 23% in Aglianico wine. Generally, for these three CTs (TR4, TG, and TB), as the wine polyphenolic

content increased, the astringent capacity of these tannins was quite small. This phenomenon seems not to be dependent on the tannin type but on the polyphenolic matrix of wines. In fact, TR4 (a condensed tannin mixture), TP (an ellagitannin mixture), and TG (a gallotannin mixture) showed a similar effect on the same wines but with a different order of astringency: TR4 > TG > TP. From these data it can be argued that wine polyphenols such as anthocyanins and flavanols may effectively interact with isogenous CTs influencing the astringency of the treated wine. Depending on the wine, different possible adducts can be formed between CTs and anthocyanins and flavanols, that favour colour stabilisation and structure complexity. In conclusion, SPI has provided an objective evaluation of the astringency of CTs in model solutions and wines. SPI has represented a useful tool to differentiate between commercial preparations of tannins of different origins. Condensed tannin resulted in more astringency than hydrolyzable tannins. The addition of CTs in wine does not necessarily cause an increase in astringency; it depends on wine type. In the case of Aglianico wine the astringency was not enhanced. It may be postulated that the matrix may interact with isogenous tannins so that the richer in polyphenols the wine, the lower the effect of CTs addition on wine astringency. On the basis of these results, the type of tannins and the amount added during the refinement of different types of wine could be optimized using the SDS–PAGE-based method. This can help enologists in the choice of commercial tannin to use according to the wine and the desired result. Further studies will focus on the influence of anthocyanins and flavanols of red wines on the SPI, in order to better understand the chemical and sensorial modifications caused by CTs.

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3.2.2 Precipitation of Salivary Proteins After the Interaction with Wine: The Effect of Ethanol, pH, Fructose, and Mannoproteins

Astringency is a complex sensation mainly caused by the precipitation of salivary proteins with polyphenols. In wine it can be enhanced or reduced depending on the composition of the medium. In order to investigate the effect of ethanol, tartaric acid, fructose, and commercial mannoproteins (MPs) addition on the precipitation of salivary proteins, the saliva precipitation index (SPI) was determined by means of the sodium dodecyl sulphate polyacrylamide gel electrophoresis of human saliva after the reaction with Merlot wines and model solutions. Gelatin index, ethanol index, and Folin–Ciocalteu index were also determined. As resulted by Pearson's correlation, data on SPI were well correlated with the sensory analysis performed on the same samples. In a second experiment, increasing the ethanol (11%–13%–17%), MPs (0–2–8 g/L), fructose (0–2–6 g/L) level, and pH values (2.9–3.0–3.6), a decrease in the precipitation of salivary proteins was observed. A difference in the SPI between model solution and red wine stated that an influence of wine matrix on the precipitation of salivary proteins occurred.

3.2.2.1 Introduction

Astringency may be considered the resulting of different sensations as drying, roughing, and puckering of the epithelium (Lee and Lawless 1991) felt in the mouth after the ingestion of foods and beverages containing polyphenols. This mouthfeel is localized not only in specific regions of the tongue, as it happens for the gustative sensations, but it also involves the overall buccal cavity like soft palate, gingives, and lips (Breslin *et al.*, 1993). One of the main mechanisms that have been proposed to explain astringency is based on the chemical interactions of some salivary constituents with polyphenols and on the formation of protein–polyphenol complexes. The precipitation of these complexes is able to stimulate mechanoreceptors connected with trigeminal nerve and to transmit to brain the perception of astringency. This results in tactile sensations characterized by different astringent subqualities, as described by the mouthfeel wheel (Gawel *et al.*, 2000). Other scientists considered astringency a taste because like sour, sweet, salty, and bitter compounds, astringent stimulus is able to activate signal transduction pathways (Spielman, 1990). However, the weight of evidence favors the tactile sensation as principally caused when astringent substances precipitate salivary proteins that give saliva its lubricity (Breslin *et al.*, 1993). During last decades, astringency has been largely studied in red wine, because it represents an important quality attribute. Wine is a complex matrix and its taste perception is determined by a balance among sensory active compounds like acids, sugars, ethanol, and others. All these gustatory stimuli have been shown to affect the perception of astringency. Adding acids to wines or tannic acid solutions produced an increase in astringency (Guinard *et al.*, 1986). On the contrary, the addition of sweeteners was observed to attenuate the mouth dryness typical of the astringent sensation both of tannic acid solutions (Lyman and Green, 1990) and red wine (Ishikawa and Noble, 1995). The astringent sensation can also be altered by the presence of the most important component of wine: the ethanol (Lesschaeve and Noble, 2005). As the ethanol level increased in model solution (Fontoin *et al.*, 2008), a decrease in perceived astringency and in some astringent subqualities (Vidal *et al.*, 2004a) was observed. In case of red wine, astringency sensation was affected by alcohol reduction (Meillon and others 2009). Astringency can also be modulated by the presence of some polysaccharides, such as mannoproteins (MPs), decreasing the intensity of some astringent attributes and contributing to the fullness of model wine solutions (Vidal *et al.*, 2004b). During tasting, the influence of wine components on astringency perception may involve different mechanisms that govern gustatory and somatosensory interactions and

their cross-modal modulation in the mouth. When assessing mixtures of flavor eliciting compounds, 2 levels of interaction must be taken into account: (i) psychological, considering cognitive effects of different stimuli being perceived together in the mouth; and (ii) physiological, including both chemical interactions occurring in solution which may directly affect perception and secondary interactions between one of the mixture components and the taste receptors/transduction mechanisms of the other component. From a psychological point of view, when tastants and chemesthetic substances in a matrix mix with saliva to reach the receptive areas, networks of brain areas are involved specialized for integrating different types of multimodal information (Verhagen and Engelen, 2006). As concern physiology, it was observed that viscosity of the saliva–wine solution seems to affect the way we perceive astringency after the addition of acids (Luck *et al.*, 1994), sugars (Smith *et al.*, 1996), ethanol (Pickering *et al.*, 1998), and MPs (Jones *et al.*, 2008). Until now, no extensive studies investigating the occurrence of the addition of factors such as acids, sugars, ethanol, and MPs in wine on polyphenols–salivary proteins precipitation have been carried out. Several studies focused on methods for astringency prediction, based on polyphenol–proteins interactions. Both suspended particles (Monteleone *et al.*, 2004) and insoluble complexes (Llaudy *et al.*, 2004) have been considered. Given that the strongest correlation between sensory analysis and analytical methods was obtained for the formation of insoluble complexes (Kennedy *et al.*, 2006), our attention has been focused on protein precipitation assay as an analytical method for astringency assessment. In this work, the saliva precipitation index (SPI), based on the precipitation of selected salivary proteins after the reaction with wine polyphenols (Rinaldi *et al.*, 2010), was utilized to better understand the influence of factors such as ethanol, pH, fructose, and mannoproteins on astringency of Merlot wines and model solutions. In a second experiment, the influence of the concentration of these factors on the precipitation of salivary proteins was evaluated. Because the protein precipitation method is similar to the physiological response to astringents, it seems to be a useful *in vitro* tool for understanding how modification by different factors leads to modification in astringency perception.

3.2.2.2 Materials and Methods

3.2.2.2.1 Reagents

Solvents of HPLC grade, D(-)- fructose and L(+)-tartaric acid were purchased from J.T. Baker (Levanchimica; Bari, Italy). Tannic acid was purchased by Extrasynth´ese (Lyon, France). Commercial mannoproteins were provided by Laffort (Bordeaux, France). Model solutions (S) and Merlot wines (W) The model solution (S) was composed of tannic acid 3 g/L, ethanol 13% v/V, tartaric acid 4 g/L, and pH = 3.6. Base parameters of Merlot wine (W) were: 2.95 ± 0.17 g/L gallic acid equivalent (GAE); ethanol 11% v/V; pH = 3.6; <1 g/L residual sugars. Ethanol (2%–4%–6% v/V), D(-)- fructose (2–4–6 g/L), L(+)-tartaric acid (2–4–6 g/L), and mannoproteins (2–4–8 g/L) were added to S and W. After L(+)-tartaric acid addition in S and
W, the pH was immediately measured by Crison pH-meter Basic 20. S and W samples were used for phenolic, SPI, and sensory analysis.

3.2.2.2.2 Phenolic analysis

Total phenolics (Folin–Ciocalteu index (FCI)) were measured according to Waterhouse (2001) and were expressed in g/L of GAE. Gelatin index was evaluated according to Glories methods (1984) with some modifications; the index was calculated as the difference between the total wine phenolics by FCI and the concentration after gelatin precipitation. Results were expressed as a percentage, referring this difference to the total

phenolics concentration. The content of polyphenols combined with polysaccharides was estimated using the ethanol index (Glories 1984).

3.2.2.2.3 SPI analysis

Human saliva (HS). HS was obtained by mixing resting whole mouth saliva samples from different individuals. Saliva collection was performed for between 10 and 11 am. Subjects were asked not to consume any food and beverage for 2 h before saliva collection. Saliva was spontaneously collected from 6 nonsmoking volunteers (3 males and 3 females) by expectorating saliva into a preweighted ice-cooled tube for 5 min. The resulting mix was centrifuged at 10000g for 10 min to remove any insoluble material, and the supernatant was referred to as HS. Resting HS flow rates were determined gravimetrically and expressed as milliliter per minute (mL/min). An average resting salivary flow rate is estimated at 0.64 ± 0.08 mL/min.

Binding assays. Interaction mixtures (150 μL final volume) contained 100 μL of HS and 50 μL of S or W, which were previously filtered at 0.45 μm (Millipore; Rome, Italy). Binding assays were performed in Eppendorfs maintained at 25 °C for 5 min (Sarni-Manchado and others 1999). The mixture was then centrifuged for 10 min at 10000g. The analyses were performed on the resulting supernatant. Binding assays were performed in triplicate.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) electrophoresis. The SDS–PAGE electrophoresis of HS before and after the binding assay was performed in triplicate on a Bio-Rad Protean II xi Cell electrophoresis apparatus (Bio-Rad, Milano, Italy) using a PowerPac 1000 Bio-Rad power supply set at 150 V/gel for the stacking gel and 180 V/gel for the resolving gel. Samples mixed with an equal volume of 2× electrophoresis sample buffer (0.125 M Tris–HCl, 4% SDS; 20% v/V glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8) and heated at 95 ◦C for 4 min were analyzed by SDS–PAGE using 30% acrylamide/bisacrylamide (37.5 : 1) solution. The resolving gels were 14% acrylamide, and stacking gels were 5% acrylamide. The gelswere fixedwith a mixture of ethanol, acetic acid, and deionized water (40 : 10 : 50) for 1 h. After washing in water for 5 min, the gels were stained with Coomassie Brilliant Blue R250 staining solution (Bio-Rad). The destain step was performed by incubation in the destain solution Coomassie Blue R250 (Bio-Rad). The apparent molecular weights of bands 1 and 2 were calculated from the linear regression equation of log molecular weight against mobility, by comparison with the migration rates of precision plus protein standards all blues (Bio-Rad).

3.2.2.2.4 Densitometry

Densitometric tracing of gels was performed with a Bio-Rad GS800 densitometer, and electrophoretic data were analyzed by Quantity One analysis software, Version 4.5 (Bio-Rad).

SPI. The SPI was obtained by the density reduction of the bands 1 (59–54 KDa) and 2 (15 KDa) marked with asterisk (∗) in Figure 1, as described by Rinaldi *et al.,* (2010).

3.2.2.2.5 Sensory analysis

Fifty subjects composed of enologists and winemaking experts, participated at a sensory course organized by the University of Naples “Federico II.” Potential participants were screened following IS0 guidelines (IS0 8586-1 1993). Thirty-one were chosen on the basis of their sensory capabilities, interest, and availability. Screening tasks included basic taste identification and intensity rating tests. Panelists were familiarized with

samples and tasting procedures in ten 1-h training sessions. They had been introduced first to the theory of astringency and extensively trained to differentiate astringency from bitterness and sourness using 3.0 g/L tannic acid, 0.25 g/L quinine sulfate, and 4.0 g/L tartaric acid as examples of
astringency, bitterness, and sourness, respectively. Thirty-one panelists indicated an ability to discriminate among these taste stimuli. In the following sessions, panelists were familiarized with astringency rating. They were asked to evaluate global astringency of tannin acid at different concentrations (0.5–1.0–2.5–3.5) on a 5-point scale (1 = not detected, 2 = weak, 3 = medium, 4 = strong, 5 = very strong) first in water and then in wine solution. The obtained data were used for assessing the reliability and consistency of the panelists, which were considered to be acceptable ($p < 0.05$ for reproducibility of scores of replicate samples). Successively, during 2 tasting sessions, 5 samples (W or S) were evaluated in duplicate. Each tasting session consisted of 2 sets and in each set, 5 samples were evaluated, with the order of samples randomized in each set. In each session, W samples (10 mL) were presented in balanced random order at room temperature (18±2 °C) in black tulipshaped glasses coded with 3-digit random numbers. The assessors were instructed to pour the whole sample in their mouth, hold it for 8 s, expectorate and rate the perceived overall astringency using a 5-point scale (1 = not detected, 2 = weak, 3 = medium, 4 = strong, 5 = very strong). Judges rinsed twice with deionized water, and then waited at least 30 s between samples. Samples S were evaluated in the same manner as W samples.

3.2.2.2.6 Statistical analysis
To determine the effect of ethanol, tartaric acid, fructose, and MPs addition among studied variables, one-way analysis of variance (ANOVA) analysis was used when the variables fulfilled the parametric conditions. Fisher's least significant differences (LSDs) procedure was used to discriminate among the means of the variables when necessary.When the variances were not homogeneous, data were analyzed using Kruskall–Wallis test and significant differences were established by using Notched Box Plots. Multifactorial ANOVA with second-order interactions was used to evaluate the relationships between included factors. Differences of $p < 0.05$ were considered significant. Elaborations were carried out by means of Statgraphics 5.0 Plus-PC (Manugistics, Inc., Rockville, MD, U.S.A.). All analyses were carried out in triplicate.

3.2.2.3 Results and Discussion
In the phenomenon of astringency, the interaction of polyphenols with salivary proteins in the mouth causes physical changes in the saliva, which are sensed by the mechanoreceptors in the oral cavity. Hydrogen bonding and hydrophobic interactions are the most likely mechanisms under physiological conditions (Clifford, 1986). As previously reported, by means of the SDS–PAGE, human saliva pattern changes after the binding reaction with polyphenols extracts (Gambuti *et al.*, 2006) and wine (Rinaldi *et al.*, 2010). After the interaction, some salivary constituents in the pool of salivary proteins present in mouth decreased. The precipitation with polyphenols leads to a reduction in density of 2 selected proteic bands that, having been correlated with astringency perception, represented a measure of the *in vitro* interactions occurring. The analytical data concerning with the ability of polyphenols to precipitate salivary proteins are given by the SPI. In order to understand if the addition of ethanol, tartaric acid, fructose, and commercial MPs may have an effect on the precipitation of salivary proteins, the interaction of HS with Merlot wine (W) and model solution (S) was carried out. In Figure 1A, the electrophoretic pattern of HS before (lane 1) and after the binding reaction with Merlot wine (lane 2), Merlot added of 2% v/V ethanol (lane 3), Merlot added of 4 g/L

MPs (lane 4), Merlot added of 2 g/L tartaric acid (lane 5), and Merlot added of 4 g/L fructose (lane 6), was shown. The densitometric analysis was performed on proteic bands marked with asterisk (∗), as previously reported (Rinaldi *et al.*, 2010). Density was represented by the area under the peak for each band typology, called band 1 and band 2, as shown in Figure 1B.

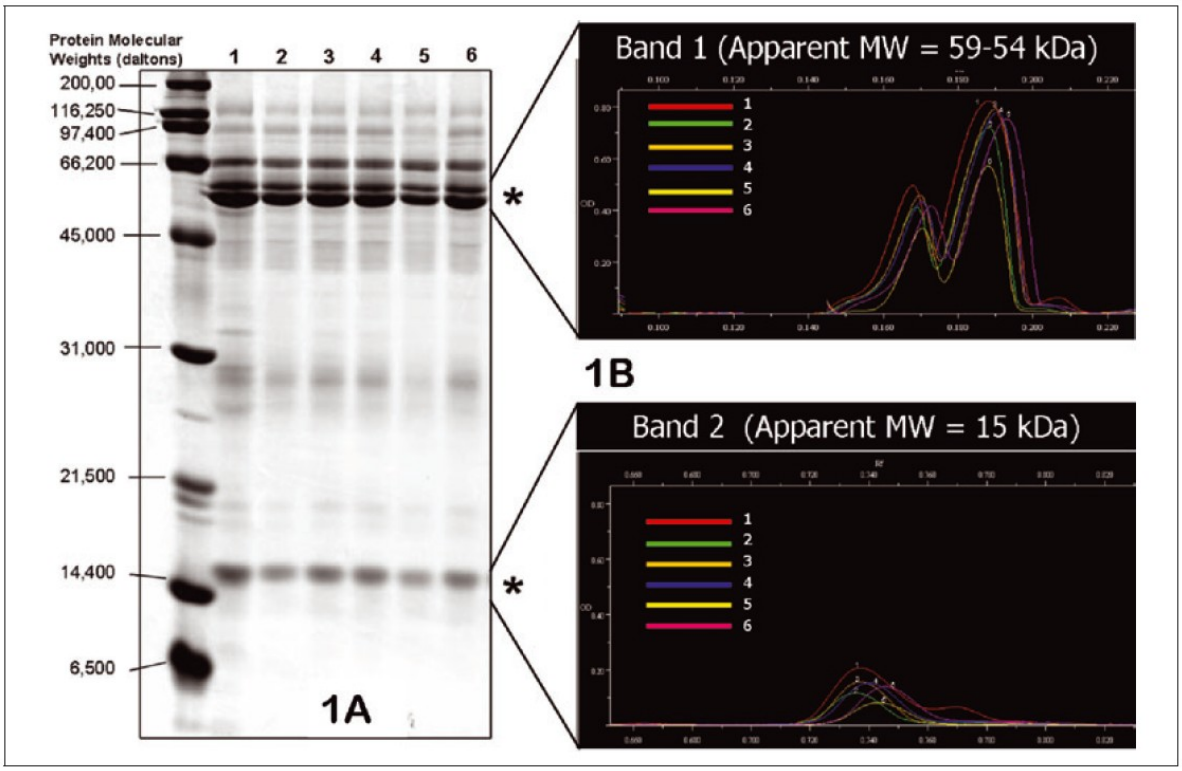

Figure 1–(A) The SDS-PAGE electrophoresis of human saliva before (lane 1) and after the binding reaction with Merlot wine (lane 2), Merlot added of 2% v/V ethanol (lane 3), Merlot added of 4 g/L MPs (lane 4), Merlot added of 2 g/L tartaric acid (lane 5), and Merlot added of 4 g/L fructose (lane 6). (B) Areas under peaks (lines 1–6 as above) for bands 1 and 2 were obtained by Biorad Quantity One Software.

The addition of tartaric acid on Merlot wine determined the most relevant decrease in density of proteic bands (Figure 1A, lane 5). As better shown in Figure 1B, in fact, the area under the yellow peaks related to wine added with acids (line 5) is less than the green one, related to control wine (line 2) for both bands. Thus, among factors considered proteins were mainly precipitated by acids. The acidic environment, in fact, promotes precipitation of salivary proteins (Payne *et al.*, 2009), increasing the binding with polyphenols. In addition, polyphenols through hydrophobic interactions could create a layer around the proteins, which may also become less hydrophilic and then precipitate. It has also been shown a direct action of tartaric acid on the formation of bigger and much more polydisperse particles of tannins (Poncet-Legrand *et al.*, 2003). In agreement with an enhanced precipitation, the SPI values significantly increased both in Merlot wine (W) and model solution (S) after the addition of tartaric acid (Table 1). Compared to control, an increase in SPI of 21.7% and 11.9% was obtained in wine and model solution, respectively. The addition of 2% v/V ethanol, on the contrary, has a slight negative effect on salivary proteins precipitation as stated by the higher density of proteic bands (Figure 1A, lanes 3) with respect to control wine (lane 2). In fact, the area under the orange peak (Figure 1B, line 3) is greater than the green one (line 2). As a consequence, values in Table 1 for SPI after the ethanol addition were significantly lower both in wine (W) and model solution (S). The percentage reduction was of 15.5% in Merlot and 14.3% in synthetic medium. The presence of this solvent can modify interactions and cause the

disruption of hydrogen bonding between polyphenols and proteins (Noble, 1990), then salivary proteins are less precipitated. Moreover, ethanol is also responsible for modification in protein folding and may induce conformational changes in proteins and in solubility of tannins (Poncet-Legrand *et al.*, 2003), thus modifying the reactivity of salivary proteins toward polyphenols. As for ethanol, the addition of 4 g/L of fructose (the reducing sugar mainly present in a not completely fermented wine) to Merlot wine determined a decrease in proteins precipitation (Figure 1A, lane 4), as well in SPI (Table 1) both in wine (17.5%) and model solution (19.7%). The hydrophilic nature of sugar may interfere with hydrogen bonding between polyphenols and protein (Ishikawa and Noble 1995), reducing the precipitation with saliva. The protective effect of carbohydrates against phenols precipitation by salivary proteins may also depend on sugar molecule that through $-CH_2OH$ substituents can accommodate with the protein without steric interference, making inaccessible the binding site to polyphenols. As regard the addition of 4 g/L of commercial MPs to Merlot wine, it seems that the binding reaction between salivary proteins and wine polyphenols was reduced or inhibited by MPs, since selected proteins were mainly precipitated after the interaction of wine without MPs (Figure 1A, lane 6). As shown in Figure 1B, the area under the pink peak (line 6) is greater than the control green one (line 2) for both bands. The SPI values in Merlot and model solution were significantly lower than controls (Table 1).

Table 1–SPI of Merlot wines (W) and model solutions (S).

	W[A]	S
Control (C)	44.33 ± 0.16c	43.26 ± 0.95b
C + Tartaric acid (2 g/L)	56.66 ± 1.61d	49.12 ± 0.49c
C + Ethanol (2% v/V)	37.46 ± 0.50a	39.12 ± 2.15a
C + Fructose (4 g/L)	36.56 ± 0.20a	34.75 ± 2.16a
C + MPs (4 g/L)	42.16 ± 0.30b	38.15 ± 0.19a

Values followed by different letters in column are significantly different ($p < 0.01$).
[A]Data were analyzed using Kruskall–Wallis test and were compared by using Notched Box Plots; values followed by different letters in row are significantly different ($p < 0.05$).

It is possible that the association of the MPs with the astringents prevents the binding with salivary proteins. As proposed by Vidal *et al.*, (2004b), physical adsorption onto the surface of polysaccharides has probably been occurred, reducing the tannic fraction reactive toward salivary proteins. Moreover, the tannin–mannoprotein combination would produce stable structures that are not reactive toward salivary proteins, explaining why wine tannins are less astringent in the presence of mannoproteins (Escot *et al.*, 2001). In order to asses if the observed effects on the precipitation of salivary proteins was perceived at a cognitive level, Merlot wines (W) and model solutions (S) added with 2% v/V ethanol, 4 g/L MPs, 2 g/L tartaric acid, and 4 g/L fructose were evaluated by sensory analysis. In Table 2, the mean sensory ratings for astringency of W and S were shown.

Table 2– Mean sensory ratings for astringency of Merlot wines (W) and model solutions (S).

	Control (C)	C + Tartaric acid (2 g/L)	C + Ethanol (2% v/V)	C + MPs (4 g/L)	C + Fructose (4 g/L)
W	3.6 ± 0.5b	4.7 ± 0.4c	2.5 ± 0.5a	2.4 ± 0.5a	2.2 ± 0.4a
S[A]	3.7 ± 0.4b	4.9 ± 0.3c	2.7 ± 0.5a	2.9 ± 0.6a	2.6 ± 0.5a

Values followed by different letters in row are significantly different ($p < 0.01$).
[A]Data were analyzed using Kruskall–Wallis test and were compared by using Notched Box Plots; values followed by different letters in row are significantly different ($p < 0.05$).

Judges perceived an increase of the sensation both in W and S after the addition of 2 g/L of tartaric acid. Previously, it was shown that acidity increased astringency as a function of pH reduction (Guinard *et al.*, 1986; De Miglio and Pickering, 2008). In the present study, the increase of astringency can be due to the lowering of pH from 3.6 to 3.2 measured after tartaric acid addition, both in W and S. This was in accordance with the observation made in model solution (Fontoin *et al.*, 2008), in which a pH decrease had a large effect on increasing astringency. On the contrary, the addition of ethanol and fructose caused a reduction in the perception of W and S astringency. The same effect on mean sensory ratings for astringency was previously observed after the addition of sugars (Ishikawa and Noble, 1995) and ethanol (Fontoin *et al.*, 2008) to wine or model solution. As regard the addition of 4 g/L of MPs in W and S, in both cases, the overall astringency was reduced. Recently, it has been shown that the addition of commercial mannoproteins in red wine (Guadalupe and Ayestaran, 2008) caused a decrease in astringency. Different and controversial reasons have been proposed to explain this phenomenon. According to Guadalupe and Ayestaran (2008), mannoproteins favorites the formation of tannin aggregates leading to a reduction of total proanthocyanidins content and a decrease in astringency. Other researchers proposed that mannoproteins interact with phenolic compounds in model wine preventing tannin aggregation at wine concentration (Riou *et al.*, 2002). The discrepancy between the proposed mechanisms by how mannoproteins can reduce astringency may be due to the different ability of these proteins to interact with red wine or model wine. From our study, it can be postulated that the interaction between salivary proteins and polyphenols is also modulated by the medium. In fact, the different composition of media utilized, the tannic acid in S and wine polyphenols in W, had a significant (F-ratio 48.95; $p = 0.0000$) influence on the precipitation of salivary proteins, as well as on the astringency perception (F-ratio = 12.24, $p = 0.0004$) evaluated by means of a multifactorial ANOVA with a second-order interaction for the variables considered at 99% confidence level. Merlot wines (W) and model solutions (S) added with 2% v/V ethanol, 4 g/L MPs, 2 g/L tartaric acid, and 4 g/L fructose were also analyzed by one of the physicochemical methodologies commonly used to evaluate astringency that is the gelatine index. In addition, the formation of polysaccharides–tannin–protein complex by the ethanol index and total phenols by FCI was also determined. In Table 3, no differences in these parameters between control and treated wines were detected, indicating that they are not sensitive to changes occurring after the addition of selected factors.

Table 3–Gelatin index, ethanol index, and Folin Ciocalteu index (FCI) of Merlot wines (W) and model solutions (S).

	Gelatin index (%)		Ethanol index (%)		FCI (g/L GAE)	
	W	S	W	S[A]	W	S[A]
Control (C)	46.5 ± 5.1a	62.9 ± 4.8a	11.5 ± 3.7a	23.4 ± 4.8a	2.95 ± 0.17a	2.20 ± 0.09a
C + Ethanol (2% v/V)	45.2 ± 2.3a	63.9 ± 3.3a	11.2 ± 3.4a	23.3 ± 1.8a	3.07 ± 0.15ab	2.25 ± 0.09a
C + Tartaric acid (2 g/L)	45.6 ± 0.3a	58.8 ± 3.7a	11.6 ± 0.4a	20.1 ± 1.3a	3.09 ± 0.07ab	1.97 ± 0.33a
C + Fructose (4 g/L)	46.6 ± 2.3a	62.3 ± 0.8a	12.6 ± 1.2a	21.3 ± 2.2a	3.08 ± 0.07ab	2.25 ± 0.13a
C + MPs (4 g/L)	48.88 ± 1.4a	64.35 ± 0.9a	11.4 ± 1.6a	22.3 ± 0.8a	3.17 ± 0.02b	2.21 ± 0.03a

Values followed by different letters in column are significantly different ($p < 0.01$).
[A]Data were analyzed using Kruskall–Wallis test and were compared by using Notched Box Plots; values followed by different letters in column are significantly different ($p < 0.05$).

As expected, FCI increased after the addition of MPs in W. This is due to the well-known reactivity of the reagent toward both sugars and proteins (Singleton and Rossi, 1965). In order to compare sensory data with the analytical data considered, a Pearson's correlation was performed. A positive correlation was found between SPI and sensory data (r = 0.888), while no correlation with gelatine index (0.017) and ethanol index (0.082) and a

negative one for FCI (−0.276) were obtained. Since the precipitation of salivary proteins was good correlated with perceived astringency, the SPI was utilized to evaluate the effect of concentration of ethanol, tartaric acid, fructose, and mannoproteins. The SPI was determined after the addition of increasing concentrations of these factors in W and S, and results were shown in Table 4.

Table 4–SPI values of Merlot wine (W) and model solutions (S) added with increasing concentration of ethanol, tartaric acid, fructose, and mannoproteins (MPs).

	Control (C) (pH = 3.6)	C + Tartaric acid (4 g/L) (pH = 3.0)	C + Tartaric acid (6 g/L) (pH = 2.9)
W	44.33 ± 0.16a	60.70 ± 2.07b	67.48 ± 1.31c
S	43.26 ± 0.95a	50.43 ± 0.26b	57.80 ± 0.89c
	Ethanol (11%)	Ethanol (13%)	Ethanol (17%)
W	47.44 ± 0.06c	44.33 ± 0.16b	35.94 ± 0.65a
S	50.28 ± 0.24c	43.26 ± 0.95b	33.25 ± 1.10a
	Control (C)	C + Fructose (2 g/L)	C + Fructose (6 g/L)
W[A]	44.33 ± 0.16c	40.96 ± 0.25b	32.95 ± 0.87a
S[A]	43.26 ± 0.95c	38.25 ± 0.29b	35.26 ± 0.13a
	Control (C)	C + MPs (2 g/L)	C + MPs (8 g/L)
W[A]	44.33 ± 0.16c	44.80 ± 0.18b	33.20 ± 1.26a
S[A]	43.26 ± 0.95c	39.52 ± 0.29b	35.99 ± 0.28a

Values followed by different letters in row are significantly different ($p < 0.01$).
[A] Data were analyzed using Kruskall–Wallis test and were compared by using Notched Box Plots; values followed by different letters in row are significantly different ($p < 0.05$).

Adding increasing amounts of tartaric acid, the pH of wines and solutions decreased from 3.6 to 2.9, determining an increase in the precipitation of salivary proteins. Several phenomena can explain these data. Poncet-Legrand *et al.*, (2003) observed a decrease of tannins solubility when the concentration of tartaric acid in a model ethanolic solution increased. Moreover, the addition of acids increases the percentage of tannins in the phenolate form, and therefore increases the possibility of hydrogen bonding. It is also likely that Van der Waal interactions and hydrogen bonding between proteins and polyphenols can be enhanced by the major accessibility of binding sites at low pH. The SPI augmented proportionally in W as in S. As the ethanol level was increased in W and S, the SPI decreased proportionally. The ethanol has a great influence on self-aggregation of tannins (Poncet-Legrand *et al.*, 2003) and on the precipitation of both tannic acid and wine polyphenols with salivary proteins, in agreement with recent findings demonstrating a direct inhibition of binding as the concentration augmented (De Miglio and Pickering, 2008). The addition of 2 and 6 g/L of fructose to Merlot wine determined a proportional decrease in the SPI, also in model solution. As regard mannoproteins, the SPI was assayed after the addition of different concentrations (2–8 g/L) in W and S, as shown in Table 4. The ability of MPs to interfere with proteins precipitation was linear with concentration in both media. Because the SPI diminished as the concentration increased, it is possible that the association of the MPs with the astringents prevents the binding with salivary proteins. From SPI data, it seems that the tannic acid present in S has a different reactivity toward salivary proteins in the presence of solutes as tartaric acid, fructose, and mannoproteins with respect to the whole pool of wine polyphenols. This may be ascribed to the different chemical nature of phenols in the matrix.

Tannic acid is generally present in commercial products as hydrolysable tannin containing up to 8 galloyl residues, while wine polyphenols are mainly proanthocyanidins; the structure features of their functional groups give to these molecules a different protein binding affinity. In order to assess the influence of wine matrix and concentration of each influencing factors on the SPI, a multifactorial ANOVA was carried out taking into

consideration the wine matrix and the concentration of the influencing factors, as well as the interactions between these factors. Table 5 shows the *F* ratio and *p* values obtained from the statistical analysis for the SPI.

Table 5– Results of multifactor analysis of variance for SPI of Merlot wines and model solutions, differing in matrix composition (M), after the addition of increasing concentrations (c) of influencing factors.

Influencing factors	Matrix (M)		Concentration (c)		M x c	
	F ratio	*p* values	*F* ratio	*p* values	*F* ratio	*p* values
Ethanol	0.71	0.4120	256.27	0.0000	8.59	0.0013
Tartaric acid	229.36	0.0000	272.88	0.0000	19.94	0.0000
Fructose	12.77	0.0025	145.63	0.0000	8.02	0.0017
MPs	60.34	0.0000	270.77	0.0000	54.22	0.0000

The *F* ratio represents the quotient between variability due to the considered effect and the residual variance. The *F* ratio values are also comparable in each column, because the number of observations was the same in all cases. A higher value of *F* ratio means a more marked effect of that factor on a variable. According to this, variables, in general, were affected by the different nature of wine matrix and the increasing concentration added. Among the influencing factors, matrix (M) and concentration (c) have mainly affected the SPI after the addition of increasing amounts of tartaric acid. The other variables were differently influenced by M and c. The interactions between the 2 factors (M × c) took place in all cases, which indicates that the capability of wines supplied with increasing levels of ethanol, tartaric acid, fructose, and commercial mannoproteins to precipitate salivary proteins was different depending on the polyphenolic matrix. Therefore, matrix seems to play an important role in establishing multidentate interactions with proteins, enhancing or reducing salivary proteins binding and subsequent precipitation.

3.2.2.4 Conclusions

The precipitation of salivary proteins, which was one of the mechanisms on the basis of astringency, was stated to be affected by factors as ethanol, tartaric acid, fructose, and commercial mannoproteins added in Merlot wine and model solution. Since the protein precipitation assay has been considered one of the possible methods able to reproduce the physiological response to astringents (Kennedy *et al.*, 2006), the SPI represented a useful tool to evaluate these effects. Tartaric acid addition caused the most relevant increase in salivary proteins precipitation and, as revealed by sensory analysis, in astringency perception. Ethanol, fructose, and mannoproteins addition on the contrary caused a reduction of the sensation as well in the SPI. Increasing the concentration of each influencing factors, the effect on the precipitation of salivary proteins was enhanced. This effect is influenced by polyphenolic matrix. These results provide interesting suggestions for enologists, which could modulate the astringency of red wine by: (i) leaving some residual reducing sugars (such as fructose) in red wine during winemaking of grapes rich in tannins; (ii) avoiding the lowering of pH; (iii) adding commercial mannoproteins or promoting a "*sur lie*" aging; and (iv) harvesting grapes at high technological maturity in order to obtain wines with a satisfactory alcoholic content when possible. Further studies will aim to give a deeper insight the perturbing action of each factor by means of structural analysis of the binding. Moreover, because the results of this study showed that a physiological effect occurred, it will be interesting to evaluate the contribution of

phenomena occurring at neurophysiological level, such as the multimodal interactions among tastes and tactile sensations present in a matrix.

3.2.2.5 References

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3.3 The effect of enological practices

3.3.1 Partial dealcoholisation of red wines by membrane contactor technique: influence on colour, phenolic compounds and saliva precipitation index

In this study, red wines from different grape varieties (Merlot, Aglianico and Piedirosso) and containing different alcoholic level (from 13.67 to 15.46% v/v) were treated to diminish the alcoholic percentage of 2, 3 and 5% v/v by a polypropylene hollowfibre membrane contactor apparatus. The wines were analysed before and after partial dealcoholisation. Colour indexes and phenolics were analysed by spectrophotometric methods and HPLC. Wine astringency was evaluated by an astringency index based on the ability of treated wines to precipitate salivary proteins (SPI, Saliva Precipitation Index). For all the levels of dealcoholisation studied, changes in chromatic characteristics, Folin–Ciocalteu index and vanillin reactive flavans were below the methodological errors of the analyses. A loss of malvidin 3-monoglucoside was detected after the dealcoholisation process. Decreasing the ethanol content of wines caused an increase in SPI values.

3.3.1.1 Introduction

Alcohol levels in wine have been increasing over the last decades. This is due to the tendency for vine growers toplant grape varieties that produce more sugar, as well as climate changes and improvements in viticulture and in winemaking techniques. Today, the level of alcohol in wine is an issue as consumers have become more healthconscious. For this reason, the worldwide demand for less alcoholic wines, as well as the treatments in wine industry aimed to reduce ethanol level, is growing. In view of this exigency, the EU regulation permits up to a 2% adjustment. However, in some cases, as during very warm vintages, there is the necessity of reducing the ethanol content of wine more than 2%. Several techniques have been developed to partially reduce alcohol content in wines, such as reverse osmosis (Pilipovik and Riverol, 2005) vacuum membrane distillation (Gomez-Plaza *et al*., 1999), pervaporation (Takàcs *et al*., 2007) and spinning cone column (Belisario-Sanchez *et al*., 2009). The goal of winemaking industry is to find a method able to reduce alcohol without lowering the concentration of other compounds involved in wine quality. Membrane contactors are among the most used systems in the industry to decrease the ethanol content of 1–5%. Until now, the effect of this treatment has been mainly evaluated for sensory quality (Lisanti *et al*., 2010) and for aroma volatile compounds in model wine solution (Diban *et al*., 2008). In spite of the importance of phenolic compounds for wine colour, mouthfeel sensations, ageing behaviour and their beneficial effects on human health, no data were reported on the effect of partial dealcoholisation by membrane contactors on wine phenolics and wine chromatic characteristics. An interesting result on the effect of partial dealcoholisation by reverse osmosis on mouthfeel sensations was reported: astringency, in several cases, increased (Meillon *et al*., 2009). As this sensation is mainly due to salivary proteins–polyphenols interactions (Haslam and Lilley, 1988), this effect may be ascribed to a change in phenolic composition of wine and/or to an effect exercised by ethanol on the phenomena that origin astringency. Until now, the effect of dealcoholisation by membrane process onwine polyphenols is still not known. In addition, the role of ethanol on salivary proteins–tannins interactions is controversial. Some authors showed a decrease in the perception of astringency with the increase in ethanol (Gawel, 1998; Fontoin *et al*., 2008), while a minimal effect was reported by Noble (Noble, 1998). Scinska *et al.*, (2000) supposed that ethanol decreases astringency by a masking action due to its own bitter or sweet taste. The ethanol may also act directly on saliva properties such as viscosity (Pickering *et al*., 1998) and lubricant power (Smith *et al*., 1996). It is also possible that

ethanol acts directly on the salivary proteins-tannin binding as earlier observed for BSA-tannin binding (Rinaldi *et al*., 2010). Due to the lack in knowledge on the influence of partial dealcoholisation on wine phenolics and chromatic characteristics, in this study, a partial dealcoholisation of -2, -3 and -5% of ethanol was performed on four monovarietal wines: one Merlot, one Piedirosso and two Aglianico wines. In order to investigate the effect of partial dealcoholisation on wine astringency, sensory analysis was performed and the Saliva Precipitation Index (SPI) was also evaluated.

3.3.1.2 Materials and methods

3.3.1.2.1 Wines

Wines were provided by Taburno winery (Foglianise, Benevento). They were obtained from grapes of *Vitis vinifera* cv. Piedirosso, Merlot and Aglianico, from vineyards located in the area surrounding the city of Benevento (Campania, Italy). Three wines (Piedirosso, Merlot and Aglianico) were produced in 2008 vintage, while the second Aglianico wine was from 2007 vintage. All wines were produced by a conventional winemaking procedure.

3.3.1.2.2 Wine dealcoholisation

The dealcoholisation tests were carried out in an industrial system ALCOLESS PRIMO (Enolife s.r.l. Montemesola, Taranto, Italy) equipped with a polypropylene hollow fibre membrane contactor (Liqui-Cel, Extra-Flow 4 9 28, Celgard 950) and a centrifugal pump in stainless steel (Lowara CEA 70/3/A, Q 30–80 L/min, PZ 0.37 kW). Wines were partially dealcoholised at almost -2% (d≈2), -3% (d≈2) and -5% (d≈5) v/v of ethanol. The dealcoholisation process consists of continuous cycles in which water circulating on one surface of polypropylene membrane is gradually enriched by ethanol deriving from wine circulating on the other side of the membrane. The driving force for the process is the difference in partial pressure of ethanol across the membrane. Ethanol evaporates from the surface of solution having higher partial pressure (higher concentration in alcohol), diffuses in theform of vapour through the membrane and condenses on the surface of the water, which results in the dealcoholisation of wine and the alcoholisation of water. During all the process, wine continuously circulates from an open tank to the dealcoholisation apparatus. In order to achieve the desired dealcoholisation level, the content of ethanol in treated wine was monitored during all the process until the target level was achieved. Membrane operating parameters were: wine inlet pressure 1 bar, wine outlet pressure 0.6 bar, water inlet pressure 1 bar, water outlet pressure 0.2 bar, wine flow 35 L/h, water flow 11 L/h. The mean process time needed to achieve the dealcoholisation of -2, -3 and -5% was 1, 2 and 3 h, respectively. One hectoliter of each wine was used. The dealcoholisation treatments were done at the temperature of 20 °C. Chemical parameters of wines before and after partial dealcoholisation treatments are reported in Table 1.

Table 1 Wine chemical parameters

Wine	Ethanol (v/v %) Mean ± SD	Sugar (g/L) Mean ± SD	Total acidity (g/L tartaric acid) Mean ± SD	pH Mean ± SD	Vol. acidity (g/L acetic acid) Mean ± SD	Free SO_2 (mg/L) Mean ± SD	Total SO_2 (mg/L) Mean ± SD	Dry extract (g/L) Mean ± SD
Agl.07	15.46 ± 0.11d	3.60 ± 0.07	5.2 ± 0.08	3.89 ± 0.02	0.62 ± 0.06	32.00 ± 3.20b	75.73 ± 1.85b	35.56 ± 0.90
$Agl.07_{d\approx2\%}$	13.54 ± 0.05c	4.06 ± 0.48	5.31 ± 0.04	3.86 ± 0.03	0.59 ± 0.03	34.13 ± 1.85ab	71.47 ± 1.85ab	35.09 ± 1.20
$Agl.07_{d\approx3\%}$	12.40 ± 0.32b	3.78 ± 0.18	5.31 ± 0.08	3.86 ± 0.02	0.58 ± 0.12	29.87 ± 1.85ab	68.27 ± 3.70a	35.01 ± 1.15
$Agl.07_{d\approx5\%}$	10.84 ± 0.14a	3.84 ± 0.04	5.33 ± 0.15	3.83 ± 0.06	0.55 ± 0.06	25.60 ± 3.20a	66.13 ± 3.70a	35.69 ± 0.84
Agl.08	13.81 ± 0.25d	2.90 ± 0.15	5.22 ± 0.19	3.85 ± 0.09	0.47 ± 0.02	26.67 ± 3.70	77.87 ± 1.85b	32.59 ± 1.45
$Agl.08_{d\approx2\%}$	11.65 ± 0.18c	2.88 ± 0.34	5.2 ± 0.04	3.84 ± 0.12	0.45 ± 0.06	24.53 ± 1.85	78.93 ± 1.85b	32.38 ± 0.09
$Agl.08_{d\approx3\%}$	10.52 ± 0.09b	2.85 ± 0.20	5.25 ± 0.08	3.83 ± 0.07	0.44 ± 0.03	26.67 ± 1.85	68.27 ± 1.85a	33 ± 1.07
$Agl.08_{d\approx5\%}$	8.83 ± 0.12a	2.81 ± 0.04	5.36 ± 0.19	3.8 ± 0.04	0.44 ± 0.09	24.53 ± 3.70	69.33 ± 1.85a	33.62 ± 0.07
Merlot	13.88 ± 0.06d	2.98 ± 0.12	5.15 ± 0.04	3.78 ± 0.01	0.53 ± 0.09	20.27 ± 1.85b	51.20 ± 3.20b	31.37 ± 1.91
$Merlot_{d\approx2\%}$	11.11 ± 0.27c	2.92 ± 0.11	5.15 ± 0.02	3.73 ± 0.06	0.48 ± 0.06	20.27 ± 1.85b	49.07 ± 1.85b	30.54 ± 0.87
$Merlot_{d\approx3\%}$	10.45 ± 0.31b	3.13 ± 0.12	5.19 ± 0.08	3.72 ± 0.10	0.47 ± 0.12	17.07 ± 1.85a	42.40 ± 2.08a	31.23 ± 0.60
$Merlot_{d\approx5\%}$	8.99 ± 0.20a	3.24 ± 0.54	5.25 ± 0.19	3.7 ± 0.06	0.49 ± 0.06	14.93 ± 1.85a	39.33 ± 1.62a	32.02 ± 1.23
Piedirosso	13.67 ± 0.10d	2.84 ± 0.06	4.91 ± 0.08	4 ± 0.07	0.54 ± 0.03	27.73 ± 1.85b	72.53 ± 3.70b	33.44 ± 1.56
$Piedirosso_{d\approx2\%}$	11.53 ± 0.26c	2.93 ± 0.08	4.97 ± 0.19	3.94 ± 0.03	0.54 ± 0.06	24.53 ± 1.85b	65.07 ± 1.85a	31.61 ± 1.42
$Piedirosso_{d\approx3\%}$	10.54 ± 0.17b	3.19 ± 0.31	5.03 ± 0.15	3.95 ± 0.02	0.5 ± 0.02	20.27 ± 1.85ab	66.13 ± 3.70a	33.7 ± 0.80
$Piedirosso_{d\approx5\%}$	8.41 ± 0.22a	3.09 ± 0.30	5.19 ± 0.04	3.91 ± 0.03	0.48 ± 0.09	19.20 ± 3.20a	64.00 ± 0.00a	34.62 ± 0.92

Agl.07, Agl.08, Merlot and Piedirosso: Aglianico 2007, Aglianico 2008, Merlot and Piedirosso wines before treatment; d≈2%, d≈3%, d≈5%: wines after partial dealcoholisation of almost 2, 3 and 5% v/v EtOH. Data are expressed as the arithmetic average ± SD of three replicates. For each monovarietal wine, values followed by different letters on the column are significantly different ($p < 0.05$)

3.3.1.2.3 Standard chemical analyses and spectrophotometric measurement

Standard chemical analyses (alcoholic strength by volume, reducing sugars, total acidity, pH, volatile acidity, total dry matter and total polyphenols (Folin–Ciocalteu index) were measured according to the OIV Compendium of International Methods of Analysis of Wine and Musts. Alcoholic strength measurements were performed obtaining the distillate by a DE distillation unit (Gibertini, Milan, Italy). Measurements of the alcoholic strength of the distillate and of total dry matter were determined by densimetry using an ALCOMAT2 hydrostatic balance (Gibertini, Milan, Italy). Accuracy and repeatability of hydrostatic balance were ±0.03% vol. Tannins were evaluated as described by Ribéreau-Gayon & Stonestreet (1966). Total anthocyanins and vanillin reactive flavans were determined according to Di Stefano & Guidoni (1989). Colourant intensity, d420, d520, d620, hue and gelatine index (GI) were evaluated according to Glories methods (1984). A Shimadzu UV-1800 (Kyoto, Japan) UV spectrophotometer was used for all data pertaining to the results reported in this article. Photometric accuracy was of ±0.002 Abs, and photometric repeatability was less than ±0.001 Abs. All analyses were carried out in triplicate.

3.3.1.2.4 HPLC analysis

Equipment

A HPLC Shimadzu LC10 ADVP apparatus was used (Shimadzu Italy, Milan), consisting of a SCL-10AVP system controller, two LC-10ADVP pumps, a SPD-M 10 AVP detector and an injection system full Rheodyne model 7725 (Rheodyne, Cotati, CA) equipped with a 20 µL loop. Allthe samples were filtered through 0.45-µm Durapore membrane filters (Millipore-Ireland) into glass vials and immediately injected into the HPLC system.

Anthocyanins method

For the separation and quantification of anthocyanins, a Waters Spherisorb column (250 9 4.6 mm, 4 µm particles diameter) with precolumn was used. Twenty microlitre of wine or calibration standards were injected onto the column. The HPLC solvents were: solvent A: water/formic acid/acetonitrile (87:10:3) v/v; solvent B: water/formic acid/acetonitrile (40:10:50) v/v. Zero-time conditions were 94% A and 6% B, after 15 min the pumps were adjusted to 70% A and 30% B, at 30 min to 50% A and 50% B, at 35 min to 40% A and 60% B, at 41 min, end of analysis, to 94% A and 6% B. This zero-time solvent mixture was followed by 10-min equilibrium period prior to inject the next sample. The flow rate

was 0.80 mL/min. Detection was carried out by monitoring the absorbance signals at 518 nm. Detector sensitivity was 0.01 absorbance units full scale (AUFS). For calibration, the external standard method was used: the calibration curve was plotted for the malvidin-3-monoglucoside on the basis of peak area. The calibration curve was obtained by injecting 5 solutions (in triplicate) containing increasing concentrations of malvidin-3-monoglucoside. The anthocyanins concentrations were expressed as mg/L of malvidin-3-monoglucoside. The analyses were carried out in triplicate.

3.3.1.2.5 SPI Analysis
The SPI analysis was performed as reported by Rinaldi *et al*., (2010).

3.3.1.2.6 Statistical analysis
All the data are expressed as the arithmetic average ± standard deviation of three replicates (two in the case of sensory data). To determine the influence of dealcoholisation treatment among variables studied, one-way ANOVA analysis was used when the variables fulfilled the parametric conditions. Fisher's least significant differences (LSD) procedure was used to discriminate among the means of the variables when necessary. When the variances were not homogeneous, data were analysed using Kruskall–Wallis test and significant differences were established by using Notched Box Plots. Differences of $p<0.05$ were considered significant. Elaborations were carried out by means of Statgraphics 5.0 Plus-PC (Manugistics, Inc.).

3.3.1.3 Results and discussion
3.3.1.3.1 Anthocyanins and chromatic characteristics
A significant modification in the content of several monomeric anthocyanins was observed after partial dealcoholisation of wines ($p<0.05$) (Table 2). The loss of total monomeric anthocyanins after the partial dealcoholisation of 5% was higher for Merlot (loss of 57%; $p<0.05$) and Piedirosso (loss of 52%; $p<0.05$) than Aglianico 2007 (loss of 49%; $p<0.05$). No significant change for Aglianico 2008 wine was detected. The loss of monomeric anthocyanins was uninfluenced by their chemical nature: pigments with higher degree of methoxylation (malvidin 3-glucoside) showed behaviour similar to that observed for cyanidin 3-glucoside which contains more hydroxyls. A different trend was observed for total anthocyanins: they did not change after the dealcoholisation treatments for all red wines considered (Table 2).

Table 2 Effect of partial dealcoholisation on monomeric anthocyanins and chromatic characteristics (mean ± SD) of experimental wines

Wine	Dp3glc (mg/L)	Cy3glc (mg/L)	Pt3glc (mg/L)	Pn3glc (mg/L)	Mv3glc (mg/L)	Pn3acglc (mg/L)	Mv3acglc (mg/L)	Pn3cmglc (mg/L)
Agl.07	17.36 ± 0.41bc	20.34 ± 0.94c	26.92 ± 0.78c	9.05 ± 0.65c	230.86 ± 14.12c	0.41 ± 0.03	4.51 ± 0.56a	26.09 ± 1.73
$Agl.07_{d\approx 2\%}$	14.49 ± 0.23a	13.40 ± 0.49b	20.53 ± 1.46b	6.33 ± 1.18b	158.21 ± 4.53b	1.40 ± 0.23	9.79 ± 1.12b	14.24 ± 0.08
$Agl.07_{d\approx 3\%}$	18.09 ± 0.27c	11.47 ± 0.58a	14.82 ± 0.38a	4.36 ± 0.31a	103.34 ± 5.43a	0.83 ± 0.45	9.45 ± 0.64b	1.51 ± 0.16
$Agl.07_{d\approx 5\%}$	16.74 ± 0.72b	10.52 ± 0.66a	13.41 ± 0.30a	3.91 ± 0.32a	94.03 ± 6.15a	0.66 ± 0.11	8.73 ± 0.78b	1.47 ± 0.10
Agl.08	16.47 ± 2.73	21.50 ± 0.75b	28.62 ± 1.77a	10.52 ± 0.75	258.51 ± 16.14b		8.61 ± 3.35	5.33 ± 2.94
$Agl.08_{d\approx 2\%}$	14.57 ± 0.30	20.43 ± 0.48ab	28.75 ± 0.66a	9.92 ± 0.17	243.19 ± 5.15ab	0.71 ± 0.11	5.40 ± 0.19	3.95 ± 0.49
$Agl.08_{d\approx 3\%}$	13.59 ± 0.10	19.11 ± 0.36a	25.97 ± 0.54a	8.63 ± 0.26	216.26 ± 6.44a		5.87 ± 0.91	2.16 ± 1.88
$Agl.08_{d\approx 5\%}$	13.87 ± 0.59	20.66 ± 1.10b	29.55 ± 0.75a	10.68 ± 2.14	234.90 ± 2.56ab		5.03 ± 0.67	3.71 ± 0.47
Merlot	13.29 ± 0.45d	9.34 ± 0.28d	11.80 ± 0.17d	3.60 ± 0.16d	77.89 ± 2.43d	0.55 ± 0.10a	5.63 ± 0.20ab	1.95 ± 0.28b
Merlot $_{d\approx 2\%}$	12.29 ± 0.04c	7.74 ± 0.23c	9.65 ± 0.09c	3.01 ± 0.04c	65.70 ± 2.51c	0.73 ± 0.13a	6.00 ± 0.28b	1.73 ± 0.26b
$Merlot_{d\approx 3\%}$	11.19 ± 0.49b	6.42 ± 0.23b	7.95 ± 0.16b	2.55 ± 0.12b	52.45 ± 1.85b	0.56 ± 0.18a	5.14 ± 0.44ab	0.84 ± 0.10a
$Merlot_{d\approx 5\%}$	8.29 ± 0.47a	3.62 ± 0.71a	4.21 ± 0.86a	1.35 ± 0.31a	28.82 ± 5.84a	0.65 ± 0.10a	4.86 ± 0.78a	0.98 ± 0.73a
Piedirosso	14.53 ± 0.43b	53.20 ± 1.78c	69.49 ± 2.64c	28.42 ± 1.15c	457.41 ± 16.75c	11.06 ± 0.70	129.39 ± 20.24	4.95 ± 0.18a
$Piedirosso_{d\approx 2\%}$	13.16 ± 0.28a	33.52 ± 1.97b	47.60 ± 0.51b	16.88 ± 0.23b	305.77 ± 19.46b	6.88 ± 4.44	59.55 ± 6.58	1.96 ± 1.11a
$Piedirosso_{d\approx 3\%}$	13.02 ± 0.14a	32.18 ± 0.58b	46.51 ± 0.28b	16.80 ± 0.26b	289.40 ± 4.77b	2.30 ± 0.46	11.35 ± 0.42	12.82 ± 1.81b
$Piedirosso_{d\approx 5\%}$	12.95 ± 0.40a	27.70 ± 1.34a	40.01 ± 0.65a	14.19 ± 0.68a	243.32 ± 10.49a	2.69 ± 0.66	10.54 ± 1.19	13.28 ± 1.30b

Wine	Mv3cmglc (mg/L)	Tot. mon. anth.(mg/L)*	Tot. anth.(mg/L)[a]	d420 (%)	d520 (%)	d620 (%)	Colourant intensity
Agl.07	3.43 ± 1.43a	336.10 ± 21.55c	302 ± 12	38.0 ± 0.4	49.5 ± 0.3	12.5 ± 0.1	18.7 ± 0.0
$Agl.07_{d\approx 2\%}$	1.54 ± 0.50a	242.80 ± 7.78b	268 ± 21	36.0 ± 3.1	51.2 ± 2.3	12.8 ± 0.8	19.4 ± 0.5
$Agl.07_{d\approx 3\%}$	21.95 ± 7.10b	175.78 ± 2.74a	284 ± 15	37.5 ± 0.1	48.9 ± 0.4	13.5 ± 0.5	18.7 ± 0.0
$Agl.07_{d\approx 5\%}$	16.27 ± 1.13b	165.73 ± 10.64a	270 ± 18	36.5 ± 1.0	49.4 ± 1.0	14.1 ± 2.0	20.5 ± 0.8
Agl.08	33.56 ± 8.56	375.96 ± 36.53	192 ± 19	39.1 ± 0.0	50.7 ± 0.4	10.2 ± 0.4	10.0 ± 0.6
$Agl.08_{d\approx 2\%}$	27.38 ± 0.51	354.29 ± 7.14	203 ± 13	38.2 ± 1.1	50.1 ± 2.4	11.7 ± 3.4	11.1 ± 0.4
$Agl.08_{d\approx 3\%}$	17.87 ± 0.95	313.29 ± 26.78	213 ± 11	39.2 ± 0.6	50.6 ± 0.3	10.2 ± 1.0	11.2 ± 0.0
$Agl.08_{d\approx 5\%}$	28.97 ± 1.64	347.40 ± 6.82	215 ± 8	38.1 ± 0.2	50.4 ± 0.9	11.4 ± 1.2	11.1 ± 0.6
Merlot	25.34 ± 3.40b	149.39 ± 0.73d	192 ± 15	37.7 ± 1.6	51.1 ± 2.9	11.1 ± 1.3	15.2 ± 0.3
Merlot $_{d\approx 2\%}$	24.29 ± 1.39b	131.14 ± 3.90c	208 ± 6	41.4 ± 1.5	45.0 ± 1.1	13.6 ± 0.3	16.3 ± 0.7
$Merlot_{d\approx 3\%}$	15.60 ± 2.79a	100.98 ± 12.73b	207 ± 9	40.4 ± 0.2	46.3 ± 0.1	13.3 ± 0.3	14.6 ± 0.3
$Merlot_{d\approx 5\%}$	11.17 ± 2.74a	63.94 ± 6.29a	207 ± 4	40.0 ± 0.8	45.7 ± 0.0	14.2 ± 0.7	16.7 ± 0.5
Piedirosso	24.62 ± 1.97b	783.21 ± 6.79d	213.8 ± 7.2	36.6 ± 0.4	52.0 ± 0.8	11.4 ± 1.2	16.0 ± 0.3
$Piedirosso_{d\approx 2\%}$	21.44 ± 2.52b	506.77 ± 12.78c	217.5 ± 9.4	36.4 ± 0.4	53.6 ± 0.5	10.0 ± 0.1	17.3 ± 0.4
$Piedirosso_{d\approx 3\%}$	16.06 ± 2.26a	430.81 ± 22.02b	216.7 ± 10.1	37.4 ± 1.0	52.4 ± 0.9	10.1 ± 0.2	17.104 ± 0.5
$Piedirosso_{d\approx 5\%}$	15.80 ± 1.25a	375.26 ± 19.81a	208.6 ± 8.0	38.0 ± 0.8	52.3 ± 0.9	9.7 ± 0.0	16.6 ± 0.3

Agl.07, Agl.08, Merlot and Piedirosso: Aglianico 2007, Aglianico 2008, Merlot and Piedirosso wines before treatment; d≈2%, d≈3%, d≈5%: wines after partial dealcoholisation of almost 2, 3 and 5% v/v EtOH.

* Sum of monomeric anthocyanins

[a] Total anthocyanins spectrophotometrically evaluated. Dp3glc = delphinidin 3-glucoside, Cy3glc = cyanidin 3-monoglucoside, Pt3glc = petunidin 3-monoglucoside, Pn3glc = peonidin 3-monoglucoside, Mv3glc = malvidin 3-glucoside, Dp3acglc = delphinidin 3-(6^{II}-acetyl)-glucoside, Cy3acglc = cyanidin 3-(6^{II}-acetyl)-glucoside, Pn3acglc = peonidin 3-(6^{II}-acetyl)-glucoside, Mv3acglc = malvidin 3-(6^{II}-acetyl)-glucoside, Pn3cmglc = peonidin 3-(6^{II}-coumaroyl)-glucoside, Mv3cmglc = malvidin 3-(6^{II}-coumaroyl)-glucoside. For each monovarietal wine, values followed by different letters on the column are significantly different ($p < 0.05$)

Changes in the concentration of wine pigments after dealcoholisation may result from several processes: (1) the oxidation of O_2 sensible compounds during the dealcoholisation process, (2) the concentration effect due to the loss of ethanol from wine and (3) the adsorption on the membrane surface. A passage of water from stripping solution to the wine should be also considered. However, it is irrelevant owing to the nearly identical vapour pressure of water over the wine and over the stripping flow (Hogan *et al*., 1998). The results of this study suggest that the subtractive processes are predominant. Differences in the entity of the loss of anthocyanins among wines were already observed in a study where a membrane treatment was performed on wine of different years (Bosso *et al*., 2001). They may be due to differences in the content of O_2 quenching compounds of each wine and/or in content of colloids acting as filtration bed on membrane surface. The dealcoholisation process did not affect the chromatic characteristics of wines (Table 2). This indicates that: (1) the contribution of copigments to wine colour is dominant respect to monomeric anthocyanins, and (2) it is unaffected by the dealcoholisation treatment. It is also likely that, during the treatment, a formation of more coloured pigment may occur due to the oxygen intake and to the loss of SO_2. Several studies have also showed that increasing the concentration of ethanol a decrease in colour and copigmentation levels of wines occurred owing to the disruptive action of ethanol on copigmentation stacks (Hermosin Gutierrez, 2003; Somers and Evans, 1979). However, the effect of ethanol on colour and copigmentation depends on the ratio between anthocyanins and related copigments found in wines (Boulton, 2001). Therefore, it is likely that the presence of more copigments in Aglianico and Piedirosso wines minimise the effect of ethanol on colour.

3.3.1.3.2 Phenolics and saliva precipitation indexes

The partial dealcoholisation process did not affect the Folin–Ciocalteu index of the analysed wines (Table 3). These results are in agreement with earlier data reporting that polyphenols are only marginally adsorbed by polypropylene membrane (Ulbricht *et al*., 2009). An increase in total phenolics was, instead, observed when an Aglianico wine was totally dealcoholised probably for the concentration effect due to the removal of ethanol (Liguori *et al*., 2010). This effect was not observed in our study because the maximum diminution of ethanol (5% v/v) was not sufficient to determine a detectable concentration of total phenolics in wine. The content of vanillin reactive flavans (VRF) did not differ in each set of samples. VRF is a parameter more sensitive to monomers and small proanthocyanidins (mean degree of polymerisation less than 4 units) than large proanthocyanidins (Vrhovsek *et al*., 2001). To give more information on the effect of dealcoholisation on the chemical nature of tannic molecules, a more specific analysis on the polymerization degree and composition of tannins must be carried out. In order to investigate the effect of dealcoholisation on astringent active phenolics, the SDS–PAGE analysis of human saliva (HS) after the interaction with experimental wines was performed. Figure 1 showed the electrophoretic pattern of HS (line 1) and HS after the reaction with Agl07 (line 2), Agl07d≈2 (line 3), Agl07d≈3, (line 4), Agl07d≈5 (5).

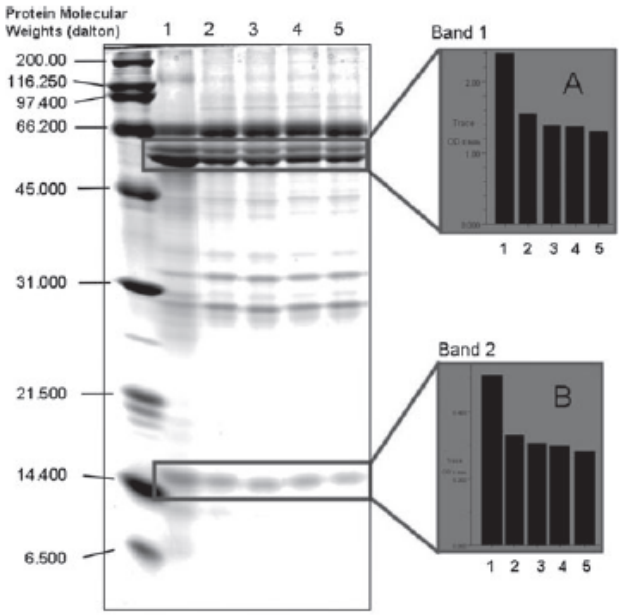

Fig. 1 SDS-PAGE of human saliva supernatant (*line* 1) and after the binding reaction with Agl07 (*line* 2), $Agl07_{d\approx2}$ (*line* 3), $Agl07_{d\approx3}$, (*line* 4), $Agl07_{d\approx5}$ (5). Optical density (mm^2) of proteic bands at 65 kDa (*Band* 1) and 15 kDa (*Band* 2)

A slight decrease in the density of proteic bands at an apparent molecular weight of 59-54 kDa (Band 1) and 15 kDa (Band 2) occurred as the dealcoholisation level of wine increased (Fig. 1). In an early study, these two bands were chosen as representative of whole salivary proteins, because they were better correlated with astringency (Rinaldi *et al*., 2010). Based on apparent molecular weight and on the comparison with mobility of pure standard (Fluka EC label 10092, Sigma, Milan, Italy), the proteic band 1 may be a-amylase, a major protein component of the HS pattern (Beeley *et al*., 1991). Band 2 may be ascribed to the acidic proline-rich phosphoproteins PRP1 identified by isolation

(Oppenheim *et al*., 1971) and by the determination of molecular masses by RP-HPLC–ESI–MS, MS/MS and MALDI-TOF (Soares *et al*., 2011). The histograms A and B are related to the density of the Band 1 and Band 2, respectively, expressed as trace quantity ($ODxmm^2$) (Fig. 1). The diminution of the density of both bands indicated that in treated wines (lines 3, 4 and 5) there are more polyphenols precipitated with salivary proteins. The SPI, an index obtained from the percentage reduction in density of Band 1 and Band 2 after interaction of saliva with wines (Rinaldi *et al*., 2010), was determined. Higher values of SPI at increasing dealcoholisation level were also observed for Aglianico 2008, Merlot and Piedirosso wines (Fig. 2).

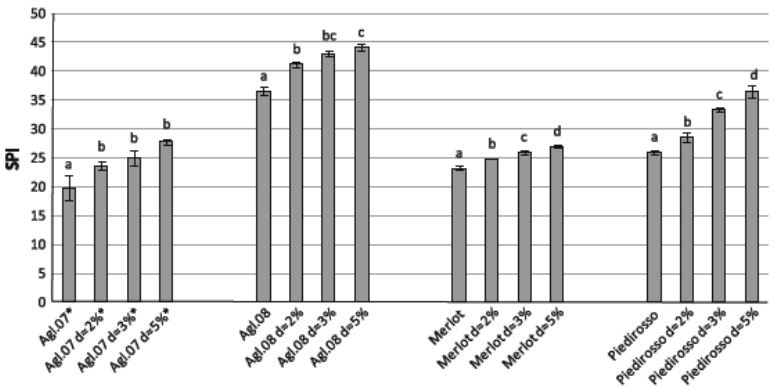

Fig. 2 Effect of partial dealcoholisation on SPI of Aglianico Merlot and Piedirosso wines. Data showing different letter are significantly different ($p < 0.05$). *Asterisk* These data were analysed using Kruskall–Wallis test ($p = 0.0188$) and were compared by using Notched *Box Plots*; values followed by different *letters* on the column are significantly different ($p < 0.05$)

These data were in agreement with sensory result reported by Meillon *et al.,* (2009) for Merlot wine after the partial dealcoholisation of -1.5 and -3%. Because the Folin–Ciocalteu index and VRF did not change among wines (Table 3), these results may indicate both a change in structure of high molecular weight tannins (molecular size and/or monomeric composition) and/or a direct influence of ethanol on the reactivity of wine tannins towards saliva. About the first hypothesis, more detailed information may derive from the analysis of molecular structure of tannins.

Table 3 Effect of partial dealcoholisation on total phenolics and vanillin reactive flavans (VRF) of experimental wines

Wine	Total phenolics (Folin–Ciocalteu index) Mean ± SD	VRF (g/L) Mean ± SD
Agl.07	71.1 ± 3.2	1.47 ± 0.10
$Agl.07_{d\approx2\%}$	64.8 ± 4.4	1.56 ± 0.16
$Agl.07_{d\approx3\%}$	65.6 ± 10.0	1.31 ± 0.14
$Agl.07_{d\approx5\%}$	73.2 ± 10.0	1.44 ± 0.06
Agl.08	75.6 ± 0.6	1.39 ± 0.11
$Agl.08_{d\approx2\%}$	74.1 ± 1.6	1.59 ± 0.14
$Agl.08_{d\approx3\%}$	75.6 ± 0.9	1.64 ± 0.17
$Agl.08_{d\approx5\%}$	76.9 ± 0.3	1.57 ± 0.20
Merlot	85.8 ± 10.0	1.39 ± 0.09
$Merlot_{d\approx2\%}$	84.8 ± 8.0	1.40 ± 0.21
$Merlot_{d\approx3\%}$	78.0 ± 2.5	1.44 ± 0.18
$Merlot_{d\approx5\%}$	74.5 ± 4.7	1.39 ± 0.13
Piedirosso	64.8 ± 0.6	1.39 ± 0.05
$Piedirosso_{d\approx2\%}$	67.2 ± 0.3	1.41 ± 0.24
$Piedirosso_{d\approx3\%}$	67.1 ± 0.5	1.45 ± 0.09
$Piedirosso_{d\approx5\%}$	63.2 ± 0.4	1.51 ± 0.17

Agl.07, Agl.08, Merlot and Piedirosso: Aglianico 2007, Aglianico 2008, Merlot and Piedirosso wines before treatment; d≈2%, d≈3%, d≈5%: wines after partial dealcoholisation of almost 2, 3 and 5% v/v EtOH. For each monovarietal wine, values followed by different letters on the column are significantly different ($p < 0.05$)

Concerning the effect on the precipitation of salivary proteins by tannins, it has been recently showed that ethanol directly influences this interaction both in model solution and red wine (Rinaldi *et al*., 2011). As the binding between tannins and salivary proteins takes place via the intermediation of hydrogen bonds between phenolic groups of tannins and peptide links of proteins and via hydrophobic interactions (Haslam and Lilley, 1988; Spencer *et al*., 1988), this effect may be due to the perturbation of these chemical links by ethanol. This is in agreement with the finding that interactions between proteins and phenolics are inhibited by the presence of hydrophobic solvents or hydrogen bond acceptor solvents (Asano *et al*., 1982; Spencer *et al*., 1988). In addition, a significant effect of ethanol on red wine tannins-protein (BSA) interactions was detected (Serafini *et al*., 1997). Therefore, ethanol could modify the native configuration of salivary proteins and/or disrupt the hydrophobic interactions causing a reduction in the ability of protein to bind tannins. The entity of the dealcoholisation effect on SPI differs among wine type (Aglianico, Merlot and Piedirosso). This may be related to other factors influencing wine astringency, such as polyphenols concentration, pH and organic acids nature and concentration (Fontoin *et al*., 2008; Smith *et al*., 1996; Guinard *et al*.,1986).

3.3.1.4 Conclusion

The partial dealcoholisation of 2, 3 and 5% v/v of Aglianico, Merlot and Piedirosso wines by a polypropylene hollow fibre membrane contactor apparatus did not change Folin–Ciocalteu index and VRF content of all red wines considered. Only a loss of monomeric anthocyanins was observed probably due to the adsorption on membrane surface and/or to the oxidation of wine when in contact with air during the treatment. The magnitude of anthocyanins loss was different among wines, likely due to differences in the content of O_2 quenching compounds in each wine. Anyway, the loss of monomeric anthocyanins did not affect the colour parameters of wines. In order to minimize these losses, dealcoholisation should be carried out under conditions limiting the dissolution of oxygen and using wines with an adequate content of molecular SO_2. SPI, an index that mimic the interaction between salivary proteins and wine tannins, increased with dealcoholisation level. The effect of dealcoholisation on SPI was significant with a loss of ethanol of only 2% v/v. Naturally, besides salivary protein precipitation, for a more complete estimation of the effect of dealcoholisation on astringency, it is necessary to investigate other factors such as changes in molecular structure of tannins. In conclusion, the partial dealcoholisation of wines by membrane contactor is a suitable technique for wine industry because, apart a loss of monomeric anthocyanins, it did not change significantly the main spectrophotometrically evaluable phenolics and the colour of red wine. Nevertheless, the oenologists have to consider that the decrease in alcohol level may affect wine astringency.

3.3.1.5 References

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3.3.2 Use of patatin, a protein extracted from potato, as alternative to animal proteins in fining of red wine

The use of plant-derived proteins as wine fining agent has gained increased interest owing to the potential allergenicity of animal proteins in susceptible subjects. Patatin P is the name of a family of glycoproteins that can be recovered from potato aqueous by-product. In this study, a comparative fining trial simulating industrial procedures with 10, 20 and 30 g/hL of commercial preparations of patatin, potassium caseinate, gelatin and egg albumin on an Aglianico (*Vitis vinifera* L.) red wine was performed. Color indexes and phenolics were analyzed by spectrophotometric methods and HPLC. The potential astringency has been evaluated by an index based on the ability of wine to precipitate salivary proteins (SPI, Saliva Precipitation Index). Patatin is a suitable alternative to animal proteins used as fining agent because: (i) a decrease in total phenolics and tannins after the treatments with 10, 20 and 30 g/hL of commercial preparation containing P was detected; (ii) Patatin, as well as all the fining agents used in this experiment, is able to diminish astringency and the content of red wine phenolics able to react with salivary proteins. Considering all concentrations tested, the effectiveness in reducing proteins reactive towards wine polyphenols was patatin = gelatine[egg albumin[casein ($p<0.05$); (iii) at each concentration considered, the treatment with patatin causes no depletion of chromatic characteristics of red wine although a significant slight loss of individual anthocyanins was observed.

3.3.2.1 Introduction

Many food proteins can act as antigens in sensitive subjects provoking allergenic reactions. Among them egg, gluten, milk, fish, crustacean shellfish, soy and nuts are the most common. These are all the important food protein sources, the major part of which has been also used as emulsifiers, gelling and foaming agents in food systems. Although potato is the largest vegetable crop worldwide, amounting to approximately 329 million metric tons annually (FAO 2009), allergy towards this tuber is much less common (Castells *et al*., 1986). Potato tubers comprise about 2 % of nitrogen compounds on a fresh basis, the 35–75 % of which is represented by proteins (Knorr *et al*., 1977; Pots *et al*., 1999). In accordance with US Food and Drug Administration, potato proteins are intended for use for a variety of functional effects associated with proteins, for example, as water binder in meat and sausage, as foaming aid in confectionary, bakery and dairy products, and as emulsifier in spreads, sauces, desserts and dressings. The described use of coagulated potato protein and hydrolyzed coagulated potato protein has been shown to be Generally Recognized as Safe (GRAS) (GRAS, 2001). Potato proteins can therefore be an interesting replacement for potentially allergenic proteins as food hydrocolloids. The major potato tuber protein (39–45 kDa) is patatin P. This is a family of glycoproteins that makes up over 40 % of the total soluble protein in potato (*Solanum tuberosum*) tubers. This potato protein might represent a suitable food additive because of its satisfactory solubility, significant foaming and emulsifying properties (Knorr *et al*., 1977; Holm andEriksen, 1980). During the last decades, industry processing potato has been expanded, exceeding considerably the amount of fresh vegetable consumation (Shieber *et al*., 2001). Tuber proteins are present in water from potato processing. Therefore, the use of these wastes as by-products for further exploitation on the production of food additives is economically attractive. Until now numerous systems for protein recovery resulted in products with unacceptable flavor and functionality. Today a satisfactory method for the recovery of proteins from potato aqueous by-product has been proposed (Bartova and Barta, 2009). A suitable use of patatin as food additive could be the fining of wine. In wine industry, the use of plant-derived proteins has gained increased interest owing to the

potential allergenicity of residual animal proteins in susceptible subjects. As concerns winemaking, several animal proteins are usually used to modulate one of most important sensory characteristic of red wine, the astringency. This is a significant descriptor of wine and an important quality attribute to the consumer. Astringency is mainly due to the interaction between salivary proteins and polyphenols such as condensed and ellagic tannins (Gambuti *et al*., 2006; Rinaldi *et al*., 2010). Proteins used for fining interact with wine tannins by a mechanism similar to that occurring during tasting. To reduce wine astringency proteins as casein, egg albumin and gelatine are used as fining agents with the risk of potential allergenicity owing to their animal origin. The potato-derived protein patatin P has an apparent molecular mass of approx. 40,000 (Park *et al*., 1983), similar to that of egg albumin. In addition, the protein P has an isoelectric point of 4.6, and the solubility is low at wine pH (Løkra *et al*., 2008). Therefore, the treatment with this protein of a red wine rich in tannins should not cause latent instability. Thus, protein P may represent an interesting vegetable alternative for wine fining. In this study, a comparative fining trial simulating industrial procedures with commercial preparation of protein P, potassium caseinate, gelatin and egg albumin on an Aglianico (*Vitis vinifera* L.) red wine was performed. This wine was chosen because Aglianico grape is rich in polyphenols and, often, wine obtained from it needs to be treated to diminish the content of astringent tannins. Because an essential requirement of a fining agent used to diminish astringency is the preservation of chromatic characteristics of wine, in this study, the ability of treated wine to precipitate salivary proteins (by SPI saliva precipitation index), phenolic composition and color has been evaluated.

3.3.2.2 Materials and methods

3.3.2.2.1 Wines

Aglianico wines were derived from 100 % Aglianico grapes obtained from vineyards located in the area surrounding the city of Benevento (Campania, Italy). Wines were produced in 2009 by Cantina del Taburno winery in agreement with standard procedure used for the production of Aglianico del Taburno DOC wine. Wines used in these experiments were not aged in barrels. Preliminary sensory tests were performed prior to the start of treatments with patatin. No contamination of wine odor and taste occurred after the addition of 10, 20 and 30 g/hL of P.

3.3.2.2.2 Fining experiments

Six months after the end of alcoholic fermentation, unfined Aglianico wine was treated with egg albumin (10, 20 and 30 g/hL), casein (10, 20 and 30 g/hL), patatin (10, 20 and 30 g/hL) and gelatin (10, 20 and 30 g/hL). All fining experiments were carried out in duplicate in 2 L bottles. The commercial preparations of patatin, egg albumin and potassium caseinate were diluted 1:10 (w/V) with distilledwater (20 °C) before to be added to the wine. The gelatin was diluted 1:10 (by volume) with distilled water (20 °C). After 1 week at 14 °C, wines were separated from lees, racked and filtered under vacuum with paper (Whatman 113 V - Dassel, Germany). After 1 year, all treated wines not showed protein instability (analysis of turbidity).

3.3.2.2.3 Enological products

Patatin P (powder) was supplied by Laffort OEnologie (Bordeaux, France). Producers guaranteed that the patatin was not from genetically modified organism. Egg albumin powder (ovoclar) was furnished by Pall Filtration & Separations (Verona, Italy), potassium caseinate powder and liquid gelatine (oliver gel 45) were supplied by Oliver Ogar (San Giovanni Lupatoto, Verona, Italy). Gelatine is derived from the acid hydrolysis of porcine collagen (bloom value = 0; isoelectric point ranging between 7 and 9).

Potassium caseinate is a salt highly soluble in water whose isoelectric point is 4.6. Patatin is obtained from raw potato tubers as reported by Lynch *et al.* (2012). The isolationmprocess involves chromatography and ultrafiltration techniques. The fractionation procedure produces two distinctnprotein fractions: a high molecular weight (HMW) fraction (> 35 kDa) and a low molecular weight (LMW) fraction (> 4 kDa, but < 35 kDa). The HMW fraction consists primarily of patatin, the main potato storage protein, whereas the LMW fraction comprises a group of protease inhibitor proteins. Successively the proteins can be fractionated to both isoelectric point and molecular weight. This allows to separate the patatin and protease inhibitor fraction. Patatin isolates are eluted at a pH of 5.8–6.2.

3.3.2.2.4 Spectrophotometric measurements

Total polyphenols (Folin-Ciocalteu) was measured according to the OIV Compendium of International Methods of Analysis of Wine and Musts. Tannins were evaluated as described by Ribéreau-Gayon & Stonestreet. Total anthocyanins and SO_2 bleaching anthocyanins were determined according to Ribéreau-Gayon and Stonestreet. Vanillin Reactive Flavans (VRF) were determined according to Di Stefano and Guidoni. Color intensity, absorbance at 420 nm, 520 nm, 620 nm (Abs420, Abs520, Abs620) and gelatin index were evaluated according to Glories methods. A Shimadzu UV-1800 (Kyoto, Japan) UV spectrophotometer was used. Ten millimeter plastic cuvettes were used. Photometric accuracy was of ±0.002 Abs and photometric repeatability was less than ±0.001 Abs. All analyses were carried out in duplicate.

3.3.2.2.5 HPLC analysis of anthocyanins

For the separation and quantification of anthocyanins, a HPLC Shimadzu LC10 ADVP apparatus was used (Shimadzu Italy, Milan), consisting of a SCL-10AVP system controller, two LC-10ADVP pumps, a SPD-M 10 AVP detector, and an injection system full Rheodyne model 7725 (Rheodyne, Cotati, CA) equipped with a 20-µL loop. A Waters Spherisorb column (250 x 4.6 mm, 4 µm particles diameter) with precolumn was used. HPLC separation of anthocyanins was carried out according to the OIV Compendium of International Methods of Analysis of Wine and Musts. Twenty microliter of wine or calibration standards was injected onto the column. All the samples were filtered through 0.45 µm Durapore membrane filters (Millipore - Ireland) into glass vials and immediately injected into the HPLC system. The HPLC solvents were as follows: solvent A: water/formic acid/acetonitrile (87:10:3) v/v; solvent B: water/formic acid/acetonitrile (40:10:50) v/v. Zero-time conditions were 94 % A and 6 % B; after 15 min, the pumps were adjusted to 70 % A and 30 % B, at 30 min to 50 % A and 50 % B, at 35 min to 40 % A and 60 % B, at 41 min, end of analysis, to 94 % A and 6 % B. This zero-time solvent mixture was followed by 10-min equilibrium period prior to inject the next sample. The flow rate was 0.80 mL/min. Detection was carried out by monitoring the absorbance signals at 518 nm. Detector sensitivity was 0.01 Absorbance units full scale (AUFS). For calibration, the external standard method was used: The calibration curve was plotted for the malvidin-3-monoglucoside on the basis of peak area. The calibration curve was obtained by injecting 5 solutions (in triplicate) containing increasing concentrations of malvidin-3-monoglucoside. The anthocyanins concentrations were expressed as mg/L of malvidin-3-monoglucoside. Calibration curve was characterized by a correlation coefficient (R^2) = 0.996. The linearity range of the calibration curve was 5–200 mg/L. Malvidin-3-monoglucoside detection limit was 0.5 mg/L. The precision of the method used was tested by six replicate analyses of a red wine sample containing 223.8 mg/L of total monomeric anthocyanins. The coefficient of variation was included between 0.9 % (for malvidin 3-monoglucoside) and 11.7 % (for malvidin 3-(6II-

coumaroyl)-glucoside) and demonstrated the good reproducibility of the HPLC analysis. The analyses were carried out in duplicate.

3.3.2.2.6 Human saliva

Whole human saliva was obtained by mixing saliva samples collected from six non-smoking volunteers (3 males and 3 females). The resulting saliva was centrifuged at 10,000g for 10 min at 4 °C and the supernatant (referred as HS) was used for the analysis. The binding assays were performed mixing 100 µL of HS and 50 µL of wines into eppendorfs maintained at 25°C for 5 min. The SDS–PAGE electrophoresis analyses were performed on the resulting supernatant (S).

3.3.2.2.7 SDS–PAGE electrophoresis

Electrophoresis was performed on a Bio-Rad Protean II xi Cell electrophoresis apparatus (Bio-Rad, Milano, Italy) using a PowerPac 1000 Bio-Rad power supply set at 150 V/gel for the stacking gel and 180 V/gel for the resolving gel. Samples (S) and fining proteins were mixed with an equal volume of 29 electrophoresis sample buffer (0.125 M Tris–HCl, 4 % SDS; 20 % v/v glycerol, 0.2 M DTT, 0.02 % bromophenol blue, pH 6.8) and were heated at 95 °C for 4 min. Successively samples were processed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 14% acrylamide resolving gels. The stacking gel was 5 % acrylamide (Bio-Rad). Both gels were fixed with a mixture of ethanol, acetic acid and deionized water (40:10:50) for 1 h. After washing in water for 5 min, the gels were stained with Coomassie Brilliant Blue R250 staining solution (Bio-Rad, #161-0436). The destain step was performed by incubation in the destain solution Coomassie Blue R250 (Bio-Rad, #161-0438).

3.3.2.2.8 Molecular weights of proteins

Molecular weights of proteins were estimated by comparison with the migration rates of SDS–PAGE molecular weight standards, broad range (Bio-Rad, #161-0317). The apparent molecular weights were calculated from the linear regression equation of log molecular weight versus mobility. The analyses were performed in duplicate. Densitometric tracing of gels was performed with a Bio-Rad GS800 densitometer. Electrophoretic data were analyzed by Quantity One analysis software, Version 4.5 (Bio-Rad). SPI determination The SPI was determined as reported by Rinaldi *et al.* (2009). The calibration curve was obtained by the analysis of optical density of two selected salivary proteins, at an apparent molecular weight of 59–54 kDa (Band 1) and 15 kDa (Band 2), in the supernatant obtained after the interaction of saliva with five standard solutions containing tannic acid (2–10 g/L in water) (Extrasynthése, Lyon, France). The linearity range was included in the range of SPI values 10–60. The correlation coefficient was (R^2) = 0.978. SPI detection limit was 5 corresponding to 0.7 g/L of tannic acid. The reproducibility of the method used was tested by seven replicate analyses of a sample of red wine. The coefficient of variation CV = 4.8 % demonstrated the good reproducibility of the analysis.

3.3.2.2.9 Total protein content in fining agents

Total protein content in egg albumin, patatin and potassium caseinate commercial preparation was measured using a colorimetric assay based on the Bradford dye-binding procedure. The protein assay reagent (Bio-Rad Laboratories, Milano, Italy) was added to diluted fining agents (1 mg/mL) and the optical density was measured at 595 nm. BSA (Sigma) was used as a standard. The protein content obtained by this method were as follows: 472.9 ± 14.1 µg/mL for egg albumin, 705.2 ± 12.3 µg/mL for patatin and 635.2 ± 41.5 µg/mL for potassium caseinate. Because Coomassie blue not react with gelatine, the

total protein content of gelatin was furnished by supplier (OLIVER OGAR, San Giovanni Lupatoto, Verona, Italy) and was 45 % (w/V).

3.3.2.2.10 Statistical analysis

All the data are expressed as the arithmetic average ± standard deviation of four replicates. To determine the influence of the concentration of fining agent used for the treatment among variables studied, one-way ANOVA analysis was used when the variables fulfilled the parametric conditions. Fisher's least significant differences (LSD) procedure was used to discriminate among the means of the variables when necessary. When data failed test for normality, they were analyzed using Kruskall–Wallis test and significant differences were established by using Notched Box Plots. Differences of $p<0.05$ were considered significant. Multifactorial ANOVA with second-order interactions was used to evaluate the relationships between concentration (10–20–30 g/hL) and fining agents (patatin, egg albumin, gelatine and casein) with SPI. Differences of $p<0.05$ were considered significant. Elaborations were carried out by means of Statgraphics 5.0 Plus-PC (Manugistics, Inc.).

3.3.2.3 Results and Discussion

3.3.2.3.1 Characteristics of protein fining agents separated by SDS–PAGE electrophoresis using Coomassie Brillant Blue as protein dye

Molecular weight distributions of fining proteins were studied by the SDS–PAGE electrophoresis. In Fig. 1, the patatin, potassium caseinate, egg albumin and gelatin patterns are shown (lane 1–2–3–4) in comparison with human saliva (lane 5). Because fining proteins are usually used to diminish astringency of red wine, and this sensation ismainly due to the precipitation of salivary proteins with wine components, the comparison with saliva proteins pattern was done in order to find similarity in molecular weight among peptides.

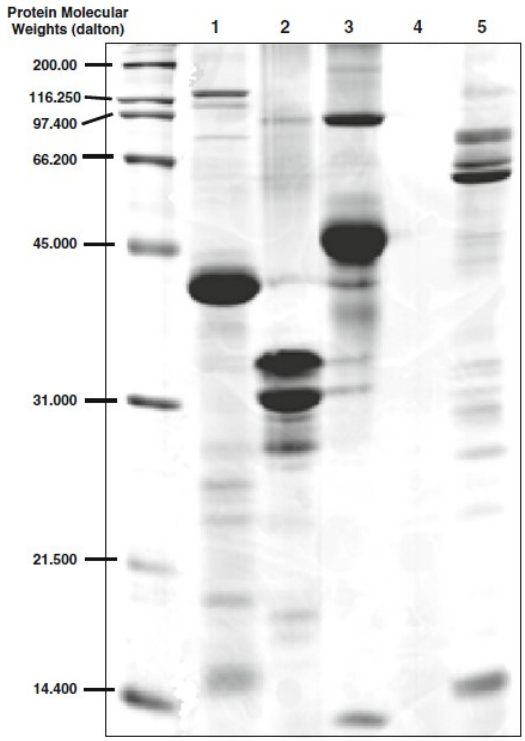

Fig. 1 SDS–PAGE analysis of proteins used for Aglianico wine fining and human saliva. *Line 1* patatin, *line 2* potassium caseinate, *line 3* egg albumin, *line 4* gelatin, line 5 human saliva. Relative molecular weight of standard proteins given on the *left side* of gel

As reported by Cosme *et al.* (2007), potassium caseinate (lane 2) presented two major bands at ≈30 and ≈34 kDa. Egg albumin (lane 3) presented the typical bands at ≈43 kDa and at 14 kDa (Cosme *et al.*, 2007; Marchal *et al.* 2002). Both egg albumin and potassium caseinate showed protein bands at ≈30 and ≈43 kDa very similar in molecular masses to the acidic PRPs of human saliva (Beeley *et al.*, 1991). PRPs are responsible for the smeary sensation in the mouth. Tannins react with PRPs reducing their amount, then the smeary taste sensation decrease, and this phenomenon is interpreted as astringency (Charlton *et al.*, 1996; Croft and Foley, 2008; Pascal *et al.* 2009). Probably the presence of similar peptides may explain the worldwide use of these two animal proteins as fining agents. No bands were detected in the molecular masses range (200–14.4 kDa) for gelatine. A similar result was already reported by Cosme *et al.* (2007). The minimal size of peptide capable of interaction with Coomassie Brilliant Blue is somewhere between penta- and non-apeptides (Tal *et al.*, 1980) and it has been previously showed that gelatin used in enology reacts weakly with Coomassie Brilliant Blue (Stoscheck, 1990). Because commercial gelatins are composed of a wide range of molecular weight proteins (Maury *et al.*, 2001)and no band was detected by the SDS–PAGEelectrophoresis analysis, it is evident that thismethod is not suitable for testing this protein fining agent. In agreement with Park *et al.* (1983) and comparable to previous results on a not well-specified protein extracted from potato (Tschiersch *et al.*, 2010), a major band at ≈40 kDa for patatin was detected (lane 1). Other bands at ≈120 kDa, between 66.20 and 97.40 kDa, between 20 and 30 kDa and at ≈15 kDa were observed. The lower molecular weight protein band present in patatin (15 kDa) was also observed in human saliva pattern (lane 5). This result is of great interest because in an earlier study, this band has been chosen, toghether with another protein band, as representative of the behavior of whole salivary proteins in the interaction with wine tannins causing astringency sensation (Rinaldi *et al.*, 2010).

However, molecular weight of proteins is not the only criterion for being an effective fining agent (Tschiersch *et al.*, 2010), and thewhole pool of chemical properties ofmacromolecules, their colloidal behavior and the real effectiveness in improving wine quality have also to be considered.

3.3.2.3.2 Effect on pigments and chromatic characteristics

A fundamental requisite for a fining treatment is that it should not cause dramatic changes in chromatic characteristics of red wine. In this study, patatin, egg albumin, potassium caseinate and gelatin at three concentration (10, 20 and 30 g/hL) have been added to Aglianico wine as fining agents. All the fining treatments, at the 3 concentrations, determined a significant loss of total individual anthocyanins and of the three main grape native anthocyanins, namely petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside (Table 1).

Table 1 Monomeric anthocyanins of Aglianico wines treated with egg albumin, potassium caseinate, patatin and gelatin

	Dp3glc Mean, SD	Cy3glc Mean, SD	Pt3glc Mean, SD	Pn3glc Mean, SD	Mv3glc Mean, SD	Vit A Mean, SD	Mv3acglc Mean, SD	Mv3cmglc Mean, SD	Total month anth. Mean, SD
Control	9.4 ± 0.3 *a	19.6 ± 4.4 *a	26.5 ± 2.4 a	13.7 ± 1.6 a	207.0 ± 11.8 a	28.4 ± 2.1 a	5.9 ± 0.5 a	22.3 ± 2.4 *a	343.8 ± 13.3 a
P10	9.2 ± 0.2 *a	14.6 ± 0.9 *ab	21.6 ± 1.6 b	11.0 ± 0.9 b	170.7 ± 11.5 b	27.0 ± 1.3 a	5.8 ± 0.5 a	17.9 ± 2.8 *ab	281.0 ± 12.1 b
P20	8.1 ± 0.9 *b	9.4 ± 1.1 *c	16.8 ± 1.9 c	8.7 ± 0.8 c	132.2 ± 14.5 c	28.8 ± 0.8 a	6.2 ± 2.3 a	12.4 ± 1.5 *b	225.1 ± 15.0 c
P30	7.9 ± 0.2 *b	13.8 ± 0.2 *b	17.6 ± 0.4 c	9.4 ± 0.3 bc	139.9 ± 4.1 c	26.4 ± 0.5 a	6.8 ± 0.5 a	11.4 ± 0.4 *b	236.3 ± 4.2 c
	$p = 0.0372$	$p = 0.0268$	$f = 26.87$, $p = 0.0000$	$f = 19.64$, $p = 0.0001$	$f = 37.39$, $p = 0.0000$	$f = 3.04$, $p = 0.0706$	$p = 0.1039$	$p = 0.0064$	$f = 45.71$, $p = 0.0000$
Control	9.4 ± 0.3 a	19.6 ± 4.4 a	26.5 ± 2.4 *a	13.7 ± 1.6 a	207.0 ± 11.8 a	28.4 ± 2.1 *a	5.9 ± 0.5 *a	22.3 ± 2.4 a	343.8 ± 13.3 a
G10	8.5 ± 0.5 ab	13.8 ± 0.7 a	19.9 ± 1.3 *b	10.1 ± 0.5 b	158.6 ± 9.7 b	27.0 ± 0.1 *ab	5.4 ± 0.5 *a	15.8 ± 1.9 b	262.1 ± 10.1 b
G20	9.4 ± 0.9 a	9.9 ± 0.2 ab	17.6 ± 0.4 *bc	8.8 ± 0.3 b	136.7 ± 2.9 bc	29.0 ± 0.5 *a	4.1 ± 1.1 *a	12.8 ± 0.5 bc	228.7 ± 3.4 c
G30	7.5 ± 0.5 b	11.9 ± 1.8 b	15.3 ± 2.2 *c	8.2 ± 1.1 b	121.6 ± 16.7 c	25.5 ± 1.4 *b	6.4 ± 0.7 *a	9.9 ± 1.6 c	209.2 ± 17.1 c
	$f = 8.71$, $p = 0.0024$	$p = 0.0044$	$f = 31.27$, $p = 0.0000$,	$f = 24.79$, $p = 0.0000$	$f = 42.64$, $p = 0.0000$	$p = 0.0087$	$p = 0.00124$	$f = 37.45$, $p = 0.0000$	$f = 47.58$, $p = 0.0000$
Control	9.4 ± 0.3 a	19.6 ± 4.4 *a	26.5 ± 2.4 *a	13.7 ± 1.6 a	207.0 ± 11.8 a	28.4 ± 2.1 ab	5.9 ± 0.5 a	22.3 ± 2.4 *a	343.8 ± 13.3 a
O10	8.4 ± 0.4 a	13.7 ± 1.2 *a	19.4 ± 1.6 *b	9.7 ± 0.7 b	154.3 ± 11.6 b	27.0 ± 0.3 b	5.4 ± 0.7 a	15.0 ± 1.4 *b	255.6 ± 11.9 b
O20	8.0 ± 1.2 a	9.7 ± 0.7 *b	17.3 ± 1.1 *b	8.9 ± 0.4 b	136.1 ± 8.4 b	29.1 ± 0.5 a	6.8 ± 1.1 a	12.4 ± 0.9 *b	232.0 ± 8.8 b
O30	8.1 ± 0.6 a	14.7 ± 2.9 *a	19.1 ± 3.6 *b	10.2 ± 1.7 b	141.6 ± 24.5 b	26.6 ± 1.3 b	6.3 ± 0.6 a	12.5 ± 2.6 *b	242.0 ± 25.2 b
	$p = 0.0751$	$p = 0.010$	$p = 0.0242$	$f = 11.78$, $p = 0.0007$	$f = 22.76$, $p = 0.0000$	$p = 0.0289$	$f = 2.67$, $p = 0.0945$	$p = 0.01205$	$f = 29.43$, $p = 0.0000$
Control	9.4 ± 0.3 a	19.6 ± 4.4 *a	26.5 ± 2.4 a	13.7 ± 1.6 a	207.0 ± 11.8 a	28.4 ± 2.1 a	5.9 ± 0.5 a	22.3 ± 2.4 *a	343.8 ± 13.3 a
C10	8.2 ± 0.2 b	14.3 ± 0.2 *a	20.4 ± 0.4 b	10.3 ± 0.4 b	161.4 ± 3.3 b	27.7 ± 0.6 a	6.1 ± 0.1 a	15.5 ± 0.4 *b	267.2 ± 3.5 b
C20	8.1 ± 0.4 b	10.0 ± 1.2 *b	17.7 ± 2.1 bc	9.1 ± 1.1 bc	139.0 ± 16.1 bc	29.6 ± 0.5 a	5.9 ± 0.8 a	12.7 ± 1.8 *b	236.6 ± 16.4 bc
C30	7.3 ± 0.1 c	12.0 ± 0.8 *b	15.5 ± 1.1 c	8.2 ± 0.6 c	124.8 ± 8.4 c	28.2 ± 0.7 a	6.0 ± 0.2 a	9.1 ± 0.7 *c	212.8 ± 8.6 c
	$f = 35.13$, $p = 0.0000$	$p = 0.0032$	$f = 32.36$, $p = 0.0000$	$f = 22.94$, $p = 0.0000$	$p = 0.0045$	$p = 0.0929$	$p = 0.9778$	$p = 0.0027$	$p = 0.0045$

P10, P20 and P30 = wines treated with patatin preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 7, 14 and 21 g/hL; G10, G20 and G30 = wines treated with gelatine preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 4.5, 9.0 and 13.5 g/hL; O10, O20 and O30 = wines treated with egg albumin preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 4.7, 9.4 and 14.1 g/hL; C10, C20 and C30 = wines treated with potassium caseinate preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 6.3, 12.6 and 18.9 g/hL. Dp3glc = delphinidin 3-glucoside, Cy3glc = cyanidin 3-monoglucoside, Pt3glc = petunidin 3-monoglucoside, Pn3glc = peonidin 3-monoglucoside, Mv3glc = malvidin 3-glucoside, VitA = vitisin A, Mv3acglc = malvidin 3-(6^{II}-acetyl)-glucoside, Mv3cmglc = malvidin 3-(6^{II}-coumaroyl)-glucoside. Egg alb. = egg albumin; K cas. = potassium caseinate. For each fining protein values followed by different letters on the column are significantly different ($p < 0.05$). * A non-parametric test was performed

The retention of part of monomeric anthocyanins by protein fining agents is in agreement with previous findings (Glories, 1984; Lovino *et al.*, 1999). After the treatments, changes in the relative distribution among monoglucosides, acetylglucosides and p-coumarylglucosides occurred. In particular, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-p-coumarylglucoside decreased in higher percentage. The effect of increasing concentration of fining agent on the decrease in monomeric anthocyanins is not the same for all proteins used. When 10 g/hL of fining agent was used, the lowest decrease in total monomeric anthocyanins (-18 %) was detected for patatin followed by potassium caseinate (-22.3 %), gelatin (-23.8 %) and egg albumin (-25.6 %). Data on gelatine are similar to previous finding showing that the fining with 8 g/hL of hydrolyzed gelatin (MW < 14.4 KDa) caused a decrease of 28 % of the content of total monomeric anthocyanins (Cosme *et al.*, 2007). The decrease in monomeric anthocyanins was higher when higher content of gelatin and potassium caseinate were used: at 30 g/hL, they removed malvidin-3-glucoside (-41.3 and 39.7 %,

respectively) and total monomeric anthocyanins (-39.1 and -38.1 %) to the greatest extent. For wines treated with patatin and egg albumin, a different trend was observed: The maximum loss was obtained at 20 g/hL (-34.5 and -32.5 %, respectively) indicating that probably a saturation effect occurred. Beside the removal of monomeric anthocyanins, a significant decrease in color intensity after fining with egg albumin and potassium caseinate was observed (Table 2).

Table 2 Chromatic characteristics of Aglianico wines treated with egg albumin, potassium caseinate, patatin and gelatin

WINE	Abs420	Abs520	Abs620	Color Intensity	Total anthoc (mg/L)	SO_2 bleaching anthocyanins (mg/L)
	Mean, SD	Mean, SD	Mean, SD	Mean, SD	Mean, SD	Mean, SD
Control	4.891 ± 0.361 *a	7.134 ± 0.625 *a	1.721 ± 0.235 *c	13.7 ± 0.1 *a	569 ± 6 *a	161 ± 2 a
P10	5.205 ± 1.204 *a	7.315 ± 1.204 *ab	1.998 ± 0.905 *b	14.4 ± 0.2 *a	561 ± 7 *a	160 ± 4 a
P20	5.368 ± 1.155 *a	7.420 ± 0.991 *b	1.669 ± 0.228 *a	14.7 ± 0.2 *a	564 ± 12 *a	159 ± 3 a
P30	5.273 ± 0.407 *a	7.212 ± 0.319 *b	1.619 ± 0.273 *b	14.5 ± 0.9 *a	558 ± 6 *a	161 ± 4 a
	$p = 0.1116$	$p = 0.0114$	$p = 0.0140$	$p = 0.0623$	$p = 0.2590$	$f = 0.37$, $p = 0.7754$
Control	4.891 ± 0.361 d	7.134 ± 0.625 *d	1.721 ± 0.235 a	13.7 ± 0.1 *c	569 ± 6 *a	161 ± 2 a
G10	4.905 ± 0.425 c	7.025 ± 0.792 *c	1.725 ± 0.080 a	13.7 ± 0.1 *c	560 ± 11 *a	157 ± 5 a
G20	5.512 ± 1.611 b	7.405 ± 0.917 *b	1.714 ± 0.130 b	14.8 ± 0.2 *b	565 ± 14 *a	155 ± 5 a
G30	5.699 ± 0.759 a	7.424 ± 0.973 *a	1.682 ± 0.078 b	15.1 ± 0.2 *a	551 ± 7 *a	159 ± 2 a
	$f = 139.4$, $p = 0.0000$	$p = 0.0027$	$p = 0.0000$	$p = 0.0076$	$p = 0.1909$	$f = 1.70$, $p = 0.2205$
Control	4.891 ± 0.361 b	7.134 ± 0.625 b	1.721 ± 0.235 b	13.7 ± 0.1 *a	569 ± 6 a	161 ± 2 ab
O10	5.368 ± 1.716 a	7.405 ± 1.695 a	1.908 ± 0.505 c	13.8 ± 0.3 *a	569 ± 7 a	165 ± 3 a
O20	4.785 ± 0.415 ab	6.901 ± 0.228 b	2.042 ± 0.645 b	13.3 ± 0.1 *b	552 ± 8 b	149 ± 2 c
O30	4.776 ± 0.545 ab	6.844 ± 0.826 b	2.114 ± 0.697 a	13.2 ± 0.1 *b	546 ± 5 b	158 ± 4 b
	$f = 14.22$, $p = 0.0003$	$f = 66.96$, $p = 0.0000$	$f = 98.88$, $p = 0.0000$	$p = 0.0041$	$f = 13.06$, $p = 0.0004$	$f = 21.95$, $p = 0.0000$
Control	4.891 ± 0.361 ab	7.134 ± 0.625 a	1.721 ± 0.235 *a	13.7 ± 0.1 a	569 ± 6 a	161 ± 2 a
C10	4.920 ± 0.251 a	7.130 ± 0.646 a	1.725 ± 0.176 *a	13.8 ± 0.1 a	562 ± 6 ab	160 ± 3 ab
C20	4.840 ± 0.249 b	7.077 ± 0.407 a	2.020 ± 0.871 *a	13.5 ± 0.0 b	562 ± 14 ab	155 ± 3 b
C30	4.844 ± 0.259 b	7.005 ± 0.462 a	2.071 ± 0.574 *a	13.5 ± 0.1 b	550 ± 11 b	163 ± 5 a
	$f = 5.50$, $p = 0.0131$	$f = 2.27$, $p = 0.1328$	$p = 0.0599$	$f = 23.67$, $p = 0.0000$	$f = 2.78$, $p = 0.0866$	$f = 3.63$, $p = 0.0452$

P10, P20 and P30 = wines treated with patatin preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 7, 14 and 21 g/hL; G10, G20 and G30 = wines treated with gelatine preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 4.5, 9.0 and 13.5 g/hL; O10, O20 and O30 = wines treated with egg albumin preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 4.7, 9.4 and 14.1 g/hL; C10, C20 and C30 = wines treated with potassium caseinate preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 6.3, 12.6 and 18.9 g/hL. For each fining protein values followed by different letters on the column are significantly different ($p < 0.05$). *A nonparametric test was performed

These data are in agreement with earlier findings showing that these protein fining agents promote a decrease in wine pigments and that the color differences can be detected visually (Cosme *et al.*, 2007). Surprisingly fining with patatin did not change color intensity and total anthocyanins of wines while an increase in Abs620 occurred. Since a decrease in monomeric anthocyanins was instead observed, it is likely that new pigments determining the shift toward higher wavelength occurred in wine during fining. The nature of these pigments may be various (Boulton, 2001). A recent study (Granato *et al.*, 2010) showing the presence of newly formed products in the pellet obtained after fining of a wine-like model solution with vegetable proteins may support this hypothesis. It is likely that, in our case, the formation of products giving rise to Abs620 took place. Fining with 20 and 30 g/hL of gelatine caused an increase in color intensity of red wine mainly due to the augmented absorbance at 420 nm. At the same time, a decrease in absorbance at 520 nm was observed. The shift to lower absorbances of the maximum of the spectrum of red wine is due to several reactions occurring during fining treatment involving phenolics, oxygen, anthocyanins and cofactor such as acetaldehyde (Timberlake and

Bridle, 1976). In accordance with Granato *et al*. (2010), acetaldehyde may be produced from the oxidation of ethanol, catalyzed either by transition metals such as iron and copper (mainly associated with plantderived proteins) or through coupled oxidation of phenols (Ross *et al.*, 2000). In order to better explain these findings, future researches will be made to determine the factors influencing the formation of pigments changing chromatic characteristics of red wine during fining and to help enologist to individuate the best fining agent and conditions to treat wine before bottling. Total phenolics, tannins, vanilline reactive flavans and astringency indexes. The effect of fining treatments at 10, 20 and 30 g/hL with ovalbumin, casein, patatin and gelatine on phenolic compounds is reported in Table 3.

Table 3 Phenolic compounds of Aglianico wines treated with egg albumin, potassium caseinate, patatin and gelatin

WINE	Total phenolics (g/L) Mean, SD	Tannins (g/L) Mean, SD	VRF (mg/L) Mean, SD
Control	2.947 ± 0.045 *a	4.106 ± 0.110 *a	1581 ± 52 *a
P10	2.662 ± 0.022 *c	3.735 ± 0.085 *ab	1563 ± 30 *a
P20	2.608 ± 0.087 *c	3.570 ± 0.126 *b	1300 ± 115 *b
P30	2.744 ± 0.058 *b	3.723 ± 0.034 *ab	1361 ± 43 *b
	$p = 0.0005$	$p = 0.01245$	$p = 0.0095$
Control	2.947 ± 0.045 a	4.106 ± 0.110 *a	1581 ± 52 *a
G10	2.590 ± 0.060 d	3.726 ± 0.089 *ab	1468 ± 74 *ab
G20	2.666 ± 0.075 c	3.559 ± 0.166 *b	1234 ± 137 *b
G30	2.835 ± 0.018 b	3.709 ± 0.061 *ab	1283 ± 83 *b
	$f = 65.93$, $p = 0.0000$	$p = 0.0198$	$p = 0.0074$
Control	2.947 ± 0.045 *a	4.106 ± 0.110 a	1581 ± 52 *a
O10	2.777 ± 0.145 *bc	3.808 ± 0.089 b	1576 ± 72 *a
O20	2.831 ± 0.025 *b	3.554 ± 0.175 c	1289 ± 142 *b
O30	2.685 ± 0.085 *c	3.664 ± 0.126 bc	1426 ± 21 *b
	$p = 0.0062$, $f = 13.59$	$p = 0.0004$	$p = 0.0086$
Control	2.947 ± 0.045 *a	4.106 ± 0.110 a	1581 ± 52 a
C10	2.670 ± 0.186 *b	3.784 ± 0.100 b	1557 ± 50 a
C20	2.758 ± 0.009 *b	3.572 ± 0.101 c	1365 ± 91 b
C30	2.769 ± 0.094 *b	3.742 ± 0.097 b	1414 ± 28 b
	$p = 0.0069$, $f = 19.07$	$p = 0.0001$, $f = 12.63$	$p = 0.0005$

P10, P20 and P30 = wines treated with patatin preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 7, 14 and 21 g/hL; G10, G20 and G30 = wines treated with gelatine preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 4.5, 9.0 and 13.5 g/hL; O10, O20 and O30 = wines treated with egg albumin preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 4.7, 9.4 and 14.1 g/hL; C10, C20 and C30 = wines treated with potassium caseinate preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 6.3, 12.6 and 18.9 g/hL. For each fining protein wine values followed by different letters on the column are significantly different ($p < 0.05$). *A non-parametric test was performed. *VRF* Vanillin Reactive Flavans

The content of total phenolics and tannins was significantly lower in wines treated with patatin, gelatin, albumin and potassium caseinate at each level of treatment considered ($p<0.05$). Similar results on total phenolics were previously found for a red wine treated with potassium caseinate, egg albumin and gelatin (Cosme *et al.*, 2009) and for Noble wine treated with casein (Sims *et al.*, 1995). The loss of tannins after the treatment with each protein ranged from 7 to 13 %. These percentages are in agreement with those reported by Maury *et al.* (2001). For all fining proteins, the maximum remotion of total phenolics and tannins was observed at 20 g/hL indicating that increasing concentration is not required for a further decrease of these compounds in wine. The fining at 10 g/hL caused no effect on vanilline reactive flavans (VRF). This analysis gives a measure of the content of monomeric flavanols and proanthocyanidins formed by 2–4 units (Vrhovsek *et al.*, 2001). Since at this concentration, a significant decrease in total tannins was instead observed it is likely that, according to literature (Maury *et al.*, 2001; Ricardo-da-Silva *et al.*, 1991; Sarni-Manchado *et al.*, 1999), the largest proanthocyanidin molecules are precipitated first in fining experiment. Inwines treated with 20 and 30 g/hL of fining

agent, the content of VRF was significantly lower than control. In particular, finingwith 20 g/hL of patatin decreased the content of low molecular weight polyphenols of ≈18 % and the percentage of loss is higher than that observed for total tannins (-13 %). The discrepancy among the effects observed at 10, 20 and 30 g/hL can be explained considering the complex nature of the colloidal equilibrium that occurs when a protein was added to a tannic solution. Beside the effect of the concentration of protein on the formation of aggregates (Siebert *et al.*, 1996), several interactions are involved and they are related to both tannins characteristics [(polymerization degree, galloylation degree, conformational flexibility and solubility (Maury *et al.*, 2001; Spencer *et al.*, 1988; Baxter *et al.*, 1997)] and protein nature [(molecular size, composition of the fractions, conformational flexibility and hydrophobicity (Oh *et al.*, 1980; Marangon *et al.*, 2010)]. It is possible that increasing the concentration of proteins, the nature of colloids formed between proteins and tannins changes and, consequently, the exposed surfaces have a higher reactivity toward vanilline reactive flavans (low molecular weight tannins). However, the knowledge of wine phenolic composition is not enough to have a prevision of astringency because of the complexity of wine solution and interaction between wine components and human response. Since the protein precipitation assays have been considered, the more appropriate methods to reproduce physiological response to astringents (Kennedy *et al.*, 2006), the SPI represents a useful tool to assess this effect. This index evaluated the precipitation of salivary proteins occurring during tasting of an astringent stimulus. SPI was determined by the SDS–PAGE analysis of human saliva (HS) after the interaction with experimental wines. Among salivary proteins migrated in gel electrophoresis, two bands (one at 59 KDa and another at 15 KDa) were chosen as representative of whole salivary proteins, because they were better correlated with astringency (Rinaldi *et al.*, 2010). The lower the value SPI, the lower the content of wine polyphenols reactive toward salivary proteins that may cause astringency. The SPI significantly differed among treated wines and controls at 10, 20 and 30 g/hL of each fining protein (Figs. 1, 2).

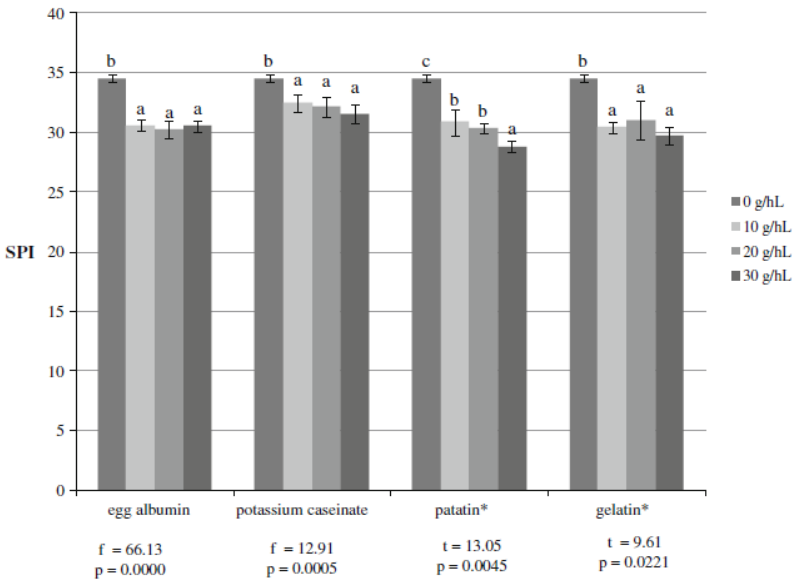

Fig. 2 Saliva Precipitation Indexes of Aglianico wines treated with egg albumin, potassium caseinate, patatin and gelatine commercial preparations. For each fining, protein values followed by *different letters* are significantly different ($p < 0.05$). *A non-parametric test was performed

After the treatment with gelatin, potassium caseinate and egg albumin no differences in SPI were detected at the 3 concentrations utilized. The minor decrease in SPI was observed for potassium caeseinate (6–9 %) while treatments with egg albumin and gelatin caused a similar effect (11.5–13 %). When increasing the concentration of patatin from 10

to 30 g/hL, a more marked decrease in polyphenols reactive toward salivary proteins was observed (from 11 to 16 %). At 30 g/hL, the efficacy of decreasing the SPI follows the order: patatin > gelatine = egg albumin > casein (F = 54.65; p = 0.0000). Amultifactorial analysis of variance (ANOVA) was carried out to assess the influence of the concentration (10–20–30 g/hL) and fining agents (patatin, gelatine, egg albumin and casein) on the precipitation of salivary proteins, as well as the interaction between these factors. The F ratio and p value were obtained fromthe statistical analysis for the SPI. The level of concentration showed a greater effect (F ratio = 105.44; p value = 0.0000) than the commercial protein preparation (F ratio = 11.57; p value = 0.0000) on SPI. The interaction between concentration and fining agent occurred (F ratio = 3.91; p value = 0.001) and was significant at 95 % of confidence. Among fining agents (considering the three concentrations), the efficacy in reducing proteins reactive toward wine polyphenols was patatin = gelatin > egg albumin > casein ($p<0.05$). Then, patatin resulted highly active in diminishing wine polyphenols reactive toward salivary proteins in the same manner as the widely used gelatin. This result could surprise because P is a glycoprotein, and several studies showed that glycoproteins have a lower affinity for tannins than non-glycosilated ones (Strumeyer and Malin, 1970; Lu and Bennick, 1998; Rowe *et al.*, 2010). However, it has been also observed that, when protein content is held constant and polyphenol concentration is increased, the precipitation increased (Siebert, 2006; Sarni-Manchado *et al.*, 2008). To find a better understanding of what happens when patatin is added to a red wine, future studies will be aimed at investigating the factors (such as protein/tannins ratio, ethanol content and pH of wine) influencing the presence of soluble and insoluble aggregates. Moreover, because several evidences indicate that hydrophobic bonding may be the major mode of interaction between condensed tannins and proteins (Siebert *et al.*, 1996; Oh *et al.*, 1980; Marangon *et al.*, 2010), this could be attributed to the relatively high exposed hydrophobicity of patatin (Creusot *et al.*, 2010). More specific experiments aimed to study the chemical–physical processes involvedmay better elucidate the nature of the phenomenon. Astringency rating furnished by a selected and trained panel is reported in Table 4.

Table 4 Mean sensory ratings for astringency of Aglianico wines treated with egg albumin, potassium caseinate, patatin and gelatin

WINE	Astringency rating Mean, SD
Control	5.9 ± 1.0 a
P10	4.5 ± 0.9 b
P20	4.7 ± 1.1 b
P30	3.8 ± 0.7 b
	$f = 19.10$, $p = 0.0000$
Control	5.9 ± 1.0 a
G10	4.3 ± 0.8 b
G20	4.5 ± 1.1 b
G30	4.5 ± 0.7 b
	$f = 6.16$, $p = 0.0012$
Control	5.9 ± 1.0 a
O10	4.2 ± 0.6 b
O20	4.3 ± 0.7 b
O30	4.2 ± 1.0 b
	$f = 6.22$, $p = 0.0012$
Control	5.9 ± 1.0 a
C10	4.5 ± 0.9 ab
C20	4.6 ± 0.9 ab
C30	4.7 ± 0.6 b
	$f = 7.74$, $p = 0.0003$

P10, P20 and P30 =wines treated with patatin preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 7, 14 and 21 g/hL; G10, G20 and G30 = wines treated with gelatine preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 4.5, 9.0 and 13.5 g/hL; O10, O20 and O30 = wines treated with egg albumin preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pureprotein are 4.7, 9.4 and 14.1 g/hL; C10, C20 and C30 = wines treated with potassium caseinate preparation at 10, 20 and 30 g/hL, respectively, corresponding values of pure protein are 6.3, 12.6 and 18.9 g/hL. Numerical ratings for astringency: absent O, very weak 1, weak 2, weak moderate 3, moderate 4, moderate strong 5, strong 6, very strong 7, extremely strong 8. Values followed by different letters are significantly different ($p < 0.05$)

Apart C10 and C20, all treatment caused a significant decrease in wine astringency, and no differences among wines treated with 10, 20 g/hL of fining agents have been observed. However, after the treatment with commercial preparations at 30 g/hL, a difference among proteins in reducing astringency was observed. In fact, panelists perceived as the less astringent the wine treated with patatin than control ($p<0.05$). This may be ascribed to the difference in protein concentration of the commercial preparation used in this study. In fact, the commercial preparation of patatin, potassium caseinate, gelatine and egg albumin contained the 70, 63, 45 and 47 % of pure protein, respectively.

3.3.2.3.3 Principal components analyses

In order to better visualizate the differences and similarities among fining treatments in all compositional parameters and their relationships with the concentration of proteins used, principal component analysis (PCA) was applied to the pooled data (Fig. 3). The first two principal components accounted for 67.66 % of the total variation. Approximately 180°angles between the vectors representing colorant intensity and SPI indicated that they were negatively correlated.

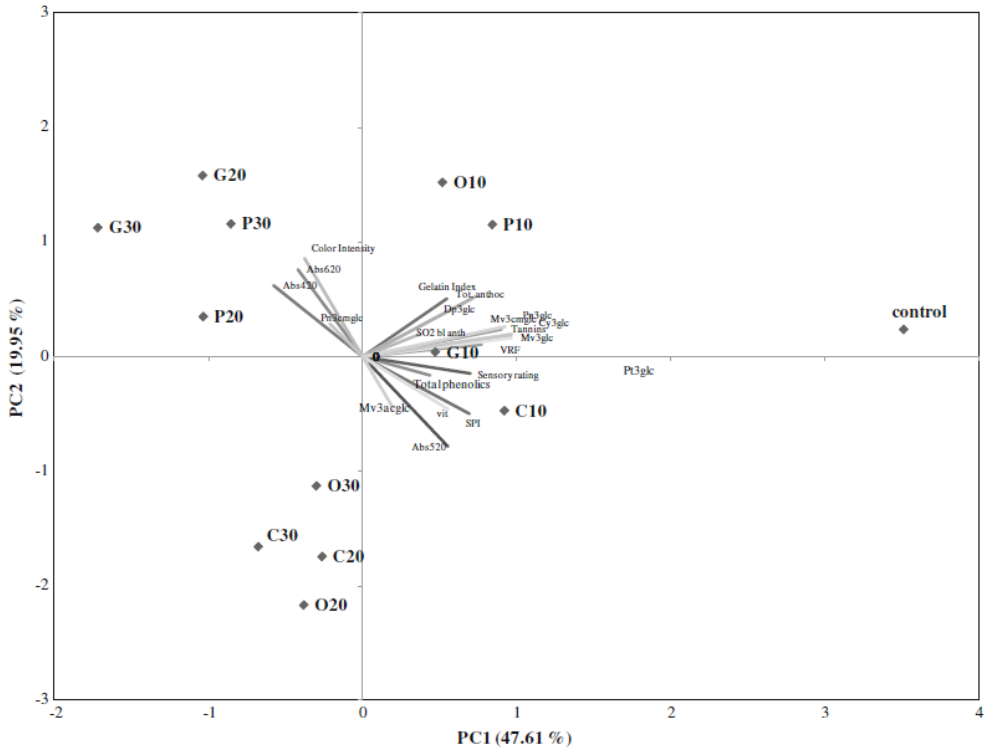

Fig. 3 PCA representation of wines (control and fined ones) and the chemical–physical variables in the plane defined by the first two principal components. O10, O20 and O30 = wines treated with egg albumin preparation at 10, 20 and 30 g/hL, respectively, these concentrations correspond to 4.7, 9.4 and 14.1 g/hL of pure protein; P10, P20 and P30 = wines treated with patatin preparation at 10, 20 and 30 g/hL, respectively, these concentrations correspond to 7, 14 and 21 g/hL of pure protein; G10, G20 and G30 = wines treated with gelatine preparation at 10, 20 and 30 g/hL, respectively, these concentrations correspond to 4.5, 9.0 and 13.5 g/hL of pure protein; C10, C20 and C30 = wines treated with potassium caseinate preparation at 10, 20 and 30 g/hL, respectively, these concentrations correspond to 6.3, 12.6 and 18.9 g/hL of pure protein; number in brackets are the pure protein concentration used. *VRF* Vanillin Reactive Flavans, SO2 bl anth = SO_2 bleaching anthocyanins. Codes of monomeric anthocyanins are reported in Table 1

The smaller angles between the vectors for colorant intensity, Abs420, Abs620 indicated a high degree of correlation. The first axis, representing 46.54 % of the total variance, was positively correlated with total anthocyanins, tannins, VRF and SPI. Wines were fairly well discriminated in function of the level of treatment along the first axis (PC1), which can thus be interpreted as a ‘‘fining’’ axis with unfined wine located on the positive side of the PC1. As the concentration increase from 10 to 30 g/hL, wines are shifted to the negative side of PC1. The second principal component (PC2), accounting for the 21.12 % of variance, is positively related to color intensity, Abs620 and Abs420, and negatively to SPI and Abs520. Wines treated with patatin were well separated along the second axis and they were discriminated on the basis of high values of colorant intensity, Abs620 and Abs420, and low values of SPI and Abs520. In particular, wines treated with 20 and 30 g/hL of patatin P were negatively correlated with sensory rating of astringency and SPI. On the contrary, wines treated with 20 and 30 g/hL of potassium caseinate and egg albumin were located in third quadrant indicating high correlation with SPI and low correlation with colorant intensity. A similar position on the graph was observed for wines treated with gelatin (20 and 30 g/hL). On the basis of these data, the commercial preparation containing patatin P is suitable for fining of red wine especially to decrease phenolic fraction reactive toward salivary proteins.

3.3.2.4 Conclusion

The results reported in this study show that patatin is a suitable non-allergenic alternative to animal proteins for fining of red wines because: (i) at each concentration considered (10, 20 and 30 g/hL), the treatment with the commercial preparation of patatin causes no depletion of chromatic characteristics of red wine although a significant slight loss of individual anthocyanins was observed; (ii) the content of total phenolics, vanillin reactive flavans and tannins was significant lower in wines treated with 20 and 30 g/hL of P; (iii) Patatin is able to diminish the content of red wine in phenolics able to react with salivary proteins. When the three concentration are considered the efficacy in reducing polyphenols reactive toward proteins was patatin= gelatine[egg albumin[casein ($p<0.05$); (iv) at each concentration considered, the treatment with preparations containing P, as well as animal proteins usually used for wine fining, caused a significant decrease in astringency. Further studies are necessary to investigate at a molecular level the binding reaction between patatin and wine tannins and to determine the factors influencing the formation of soluble and insoluble aggregates.

3.3.2.5 References

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3.4 Characterization of Aglianico (*Vitis vinifera* L. cv) grape proanthocyanidins and evaluation of their reactivity toward salivary proteins by the SPI (Saliva Precipitation Index)

Aglianico is one of the most ancient grape cultivar introduced by Greeks into Southern Italy in pre-Roman times, and is native of regions as Campania and Basilicata. The flavan-3-ol and proanthocyanidin composition of both skins and seeds of Aglianico grapes were for the first time determined by HPLC-MS. The monomeric/oligomeric and polymeric fractions were analysed for their mean degree of polymerization (mDP), percentage of galloylation (%G) and of prodelfinidin (%P) by acid-catalyzed depolymerization in the presence of phloroglucinol (phloroglucinolysis). The composition of extension and terminal units of skins and seeds fractions was determined. As a rare instance, Aglianico skin fractions present a higher amount of epigallocatechin in the terminal units. Monomers [(+)-catechin C, (-)-epicatechin EC, (-)-epicatechin-*O*-gallate ECG] and oligomers [dimers B1, B2, B3, and B4 and trimer Epi-Epi-Epi (C1)] were identified by Mass Spectrometry analysis and quantified. A comparison with international grapes as Merlot and Cabernet Sauvignon cultivated in the same geographical area and of the same year was made. In seeds fractions a content of about 180 mg/g of catechin was detected in Aglianico respect to Merlot (≈ 100 mg/g) and Cabernet Sauvignon (≈ 70 mg/g), the component present at the lowest concentration was B2 (≈17 mg/g for Aglianico and ≈ 30 mg/g for Merlot and Cabernet Sauvignon). In addition, the peak corresponding to ECG (*m/z* = 441) was detected only in Aglianico seeds. As regard skins fractions, the content of C and EC of Aglianico was similar to Cabernet Sauvignon, dimers concentration was higher than others, while only gallic acid resulted in lower amount in Aglianico. As reported by literature, the structural composition of Aglianico proanthocyanidins makes this grape cultivar a model for studying astringency. The reactivity toward salivary proteins represents the ability of tannins to precipitate proteins and then the potential astringency. For this purpose, the monomeric/oligomeric and polymeric fractions of grapes skins and seeds were dissolved in model wine at a concentration of 1 g/L and analysed by the Saliva Precipitation Index (SPI). Aglianico fractions of both skins and seeds resulted more astringent than Merlot and Cabernet Sauvignon for the high percentage of galloylation, low mDP in accordance with the sensory studies on astringency. The percentage of ECG in extension units resulted to significantly contribute to the precipitation of salivary proteins by grape tannins.

3.4.1 Introduction

Vitis Vinifera L. cv. Aglianico is a very old red grape variety of Greek origin, autochthonous of Campania and Basilicata, regions of Southern Italy that has an established wine industry. The stronger hypothesis is that during *Magna Grecia dominus* Greek settlers imported and spread this grape during the colonization of Southern Italy. Aglianico del Taburno, one of the three biotypes of Aglianico grape cultivar (Taburno, Vulture e Taurasi) is a high-quality and the best known variety used in Taburno DOCG (Denominazione di Origine Controllata e Garantita) geographical area, surrounded Benevento city. The Aglianico premium red wine produced according to the production regulation, is appreciated worldwide (Gambuti *et al.*, 2007), and the new exploited countries devoted to wine, such as California, Australia and Texas have started to commercialize it. Aglianico cultivar is a later-maturing grape and is characterized by a high content of polyphenols mainly tannins, that confer to the wine obtained from it a harsh and astringent character, if winemaking is not highly controlled. The knowledge of the structural characteristics and localization of Aglianico grape phenolics may help to understand how to better manage the vinification processes.

Polyphenols are the most important secondary metabolites and the major bioactive compounds synthesized in the berries. The most important of them are the anthocyanins, pigments responsible for the color of red grapes and young red wines, and the tannins, (proanthocyanidins) compounds responsible for astringency and bitterness of grape and wine (Cheynier *et al*., 2006; Gil-Muñoz *et al*., 2009). Variation in tannin content, composition, and polymer length are likely to determine mouthfeel and aging properties of wine (Vidal *et al*., 2003). Condensed tannins or proanthocyanidins derived from grape berries are polymers composed of terminal and extension subunits analogous to the flavan-3-ols catechin, epicatechin, epicatechin-gallate, and epigallocatechin. Subunits are linked via interflavan bonds between C-4 and C-8 and less commonly C-4 and C-6 (Haslam, 1998). In the grape skin, catechin is the primary terminal subunit, with epicatechin and epicatechin-gallate reported in much lower quantities (Souquet *et al*. 1996, Kennedy *et al*. 2001, Downey *et al.*, 2003, Monagas *et al.* 2003, Gagne *et al.,* 2006). Epicatechin is the most common extension subunit, followed by epigallocatechin and epicatechin-gallate (Souquet *et al.*, 1996; Kennedy and Jones, 2001; Monagas *et al*., 2003). The proanthocyanidin amount, composition, and mean degree of polymerization (mDP) differ between berry skins and seeds. Skin proanthocyanidins have a higher mDP and a lower proportion of galloylated subunits than those from seeds (Prieur *et al*., 1994; Souquet *et al*., 1996; Vidal *et al*., 2003). Experimental evidence has shown that the mDP and galloylation of wine proanthocyanidins are important structural variables affecting wine astringency perception (Vidal *et al*., 2003).
While the characterisation of international grape varieties such as Merlot and Cabernet Sauvignon have been extensively studied, no studies on proanthocyanidins composition of Aglianico grape cultivar have been realized. This study represents the first phytochemical compositional study of the flavan-3-ol and proanthocyanidins of Aglianico *Vitis vinifera* L. cv. The flavan-3-ol and proanthocyanidin composition of both skins and seeds of Aglianico grapes were established using high performance liquid chromatography HPLC/UV-Fluo-MS and compared with Merlot and Cabernet Sauvignon grapes cultivated in the same geographical area, the Taburno DOCG. The reactivity of the monomeric/oligomeric and polymeric grape fractions toward salivary proteins was determined by the Saliva Precipitation Index (SPI). This index obtained by a method based on the precipitation of salivary proteins by polyphenols, represents an estimation of the potential astringency of grape fractions. This may help to better understand the characteristics of grapes that are mainly correlated with the astringency perception.

3.4.2 Materials and methods

3.4.2.1 Chemicals

Phloroglucinol, (+)-catechin, (–)-epicatechin, (–)-epigallocatechin (EGC), (–)-epicatechin-3-*O*-gallate (ECG), procyanidin B1 [(–)-epicatechin-(4β-8)-(+)-catechin], procyanidin B2 [(–)-epicatechin-(4β-8)-(–)-epicatechin] were supplied from Sigma – Aldrich (Saint Quentin Fallavier, France). All solvents (HPLC grade), L-ascorbic acid and sodium acetate were purchased from Prolabo-VWR (Fontenays sous Bois, France). Procyanidin B3 [(+)-catechin-(4α-8)-(+)-catechin] and procyanidin B4 [(+)-catechin-(4α-8)-(-)-epicatechin] and trimer (C1) [(-)-epicatechin-(4β-8)-(-)-epicatechin-(4β-8)-(-)-epicatechin] were obtained from Polyphenols Biotech (Villenave d'Ornon, France).

3.4.2.2 Grape Samples and extracts

Vitis V*inifera* L. cv. Aglianico, Merlot and Cabernet Sauvignon (vintage 2010) were harvested at their technological maturity from the vineyards of "Cantine del Taburno" located in Taburno DOCG area (Foglianise, BN, Italy). About 450 g of frozen berries,

randomly selected from the collected samples of each variety (3 kg), were used for the study.
Seeds and skins were removed by hand from grapes, lyophilised for 2 days and stored at -20 °C. The frozen seeds and skins were finally ground in a ball grinder.
Five grams of the obtained powder were submitted to 6 solid/liquid extractions with 45 mL of acetone/water (80:20, v/v), followed by 2 extractions with the same volume of methanol/ water (60:40, v/v) using an accelerated solvent extraction system (DIONEX ASE 350) with the following parameters: static time set at 8 min, temperature set at 60 °C and pressure set at 150 bar. All extracts were combined and evaporated under reduced pressure at 30°C to remove organic solvents; the residue was dissolved in water and lyophilised to obtain a crude tannin extract. The extraction was made twice for each sample.

3.4.2.3 Fractionation and analyses of proanthocyanidins
The left crude tannin extract (equal to 5 g of dried powder of skins or seeds) was solubilised in 250 mL of water/ethanol (95:5, v/v) and extracted three times with chloroform to remove lipophilic material. Then the aqueous phase was extracted three times with ethyl acetate to obtain two distinctive fractions: a low molecular weight procyanidins fraction (monomeric/oligomeric tannins) in the organic phase and a high weight procyanidins fraction (polymeric tannins) in the aqueous phase. These two fractions were concentrated and lyophilised to obtain a dry powder. Fractionation of tannin extracts was repeated twice.

3.4.2.4 HPLC analyses of monomeric and oligomeric flavan-3-ols
Monomeric/oligomeric tannins extracts were solubilised in a methanol/water solution (50:50, v/v) at concentrations of 1 g/L for seed extracts and 6 g/L for skin extracts. The equipment used for HPLC analysis consisted of a Thermo-Finnigan UV–vis detector (UV–vis 200), a Thermo-Finnigan autosampler and a Thermo-Finnigan ternary pump coupled to an Xcalibur data treatment system. Separation was performed on a reversed-phase Agilent C18 (250x4 mm, 5 µm) column. The mobile phases were 50 mM dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid (solvent A), 20% solvent A with 80% acetonitrile (solvent B) and 0.2 M orthophosphoric acid adjusted with ammonia to pH 1.5 (solvent C) at a flow rate of 0.5 mL/min. Initial A and B were set at 97% and 3% respectively. The ternary mobile phase gradient was as following: 97% A and 3% B at 5 min, 92% A and 8% B at 15 min, 0% A and 8% B at 18 min, 0% A and 13% B at 30 min, 0% A and 20% B at 55 min, 0% A and 25% B at 60 min, 0% A and 30% B at 70 min, 0% A and 80% B at 75 min, 0% A and 97% B at 80 min, 97% A and 3% B from 82 to 84 min. Eluting peaks were monitored at 280 nm. Identification of mean peaks was performed by comparison to injected external standards. Calibration curves were established at 280 nm using external standards either commercial either synthesised (C, EC, ECG, B1, B2, B3, B4, C1). The results were converted in mg of dried skins or seeds weights. Analytical replicates of each sample consisted of four injections.

3.4.2.5 Determination of mean degree of polymerisation (mDP)
The proanthocyanidins mDP were determined for seed and skin extracts both in monomeric/oligomeric tannins fraction and in polymeric tannins fractions by the means of phloroglucinolysis (Drinkine *et al*., 2007). Reactions were carried out twice. Reaction products were analysed by HPLC–MS on a Hewlett–Packard 1100 series (Agilent, Massy, France) included a pump module and a UV detector and coupled to a Micromass Platform II simple quadruple mass spectrometer (Micromass-Beckman, Roissy Charles de

Gaulle, France) equipped with an electrospray ion source. The mass spectrometer was operated in negative-ion mode. The source's temperature was 120 °C, the capillary voltage was set at 3.5 kV and the cone voltage was -30 eV. Both systems were operated using Masslynx 3.4 software. The absorbance was recorded at 280 nm and mass spectra were recorded in the range of 50–1500 amu. Separation was performed on a reversed-phase Waters XTerra RR C18 (100 x4.6 mm, 3.5 µm) column at room temperature. A binary gradient system was employed using 1% (v/v) aqueous acetic acid (solvent A) and MeOH (solvent B) at a flow rate of 1 mL/min. The elution conditions were: 5% B at t0; 16% B at 1 min, 22% B at 7 min, 35% B at 8 min, 42% B at 15 min; the column was then washed with 100% B for 3 min and re-equilibrated with 5% B for 4 min before next injection.

3.4.2.6 The Saliva Precipitation Index (SPI)

The SPI method was performed as described in Rinaldi *et al*., (2012). Wine samples for the binding reaction were obtained from the dissolution of grape fraction extracts at a concentration of 1 g/L in model wine solution (ethanol 12%; pH=3.6; tartaric acid = 4 g/L).

3.4.3 Results

3.4.3.1 Chemical characterization

3.4.3.1.1 Monomeric/oligomeric fraction by HPLC/Fluo-MS analysis

For the first time the flavan-3-ol monomers (C, EC, ECG) and oligomers (B1, B2, B3, B4 dimers and a trimer C1) were identified and quantified in both seeds and skins of Aglianico grapes harvested during 2010 vintage by means of HPLC/Fluo-MS data and by comparison with the retention time and spectral features of flavan-3-ol reference compounds. The cromatografic profile of the monomeric/oligomeric fraction of Aglianico seeds was shown in Fig.1. Twelve peaks were identified that corresponded to the compounds listed in Tab. 1, with their retention time and $[M-H]^-$.

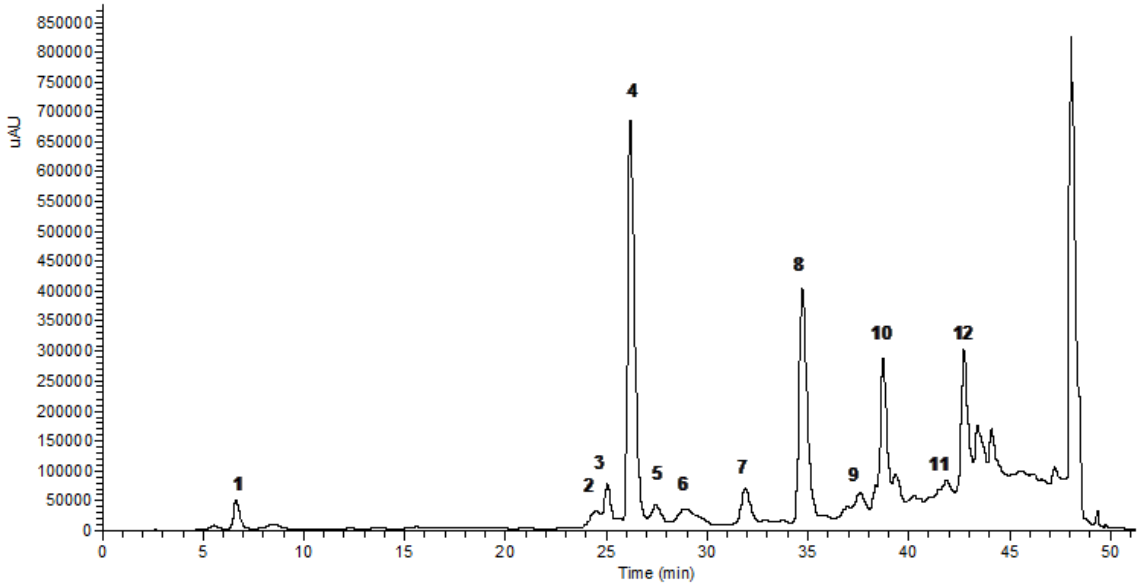

Fig. 1: Cromatografic profile of Aglianico grape seeds monomeric/oligomeric fraction.

The monomeric fraction contained as the main peaks (+)-catechin and (-)-epicatechin (4 and 8 in Fig. 1) in both seeds and skins. The ion fragment at *m*/*z* 169 corresponded to the gallic acid (peak 1). HPLC/MS analysis confirmed the presence of dimeric ($[M-H]^-$ *m*/*z* 577), dimer gallate ($[M-H]^-$ *m*/*z* 729), trimeric ($[M-H]^-$ *m*/*z* 865), and trimeric digallate ($[M-H]^-$ *m*/*z* 1169) procyanidins (Fig. 1; Tab 1). A compound with $[M-H]^-$ at *m*/*z* 441 also confirmed the presence of the monomer (-)-epicatechin-3-*O*-gallate in the oligomeric fraction of Aglianico seeds.

Tab. 1: Compounds identified in Aglianico seeds fraction by HPLC/MS, the peaks, codes, their retention time (RT) and $[M-H]^-$ (m/z).

Peak	Compound	Code	RT	m/z
1	Gallic acid	GA	6.70	169
2	(+)-catechin-(4α-8)-(+)-catechin	B3	26.40	577
3	(–)-epicatechin-(4β-8)-(+)-catechin	B1	25.08	577
4	(+)-catechin	C	26.42	289
5	Trimer	-	27.64	865
6	(+)-catechin-(4α-8)-(-)-epicatechin	B4	29.14	577
7	(–)-epicatechin-(4β-8)-(–)-epicatechin	B2	32.00	577
8	(–)-epicatechin	EC	34.95	289
9	(-)-epicatechin-(4β-8)-(-)-epicatechin-(4β-8)-(-)-epicatechin	C1	38.50	865
10	Dimer gallates	-	38.88	729
11	Trimer digallate	-	42.08	1169
12	(−)-epicatechin gallate	ECG	42.95	441

Once identified, the main compounds such as monomers (C-EC, ECG), GA, dimers (B1-B2-B3-B4), and C1 trimer were quantified both in skins and seeds extracts of Aglianico, Merlot and Cabernet Sauvignon grapes. As reported in Fig. 2, the seeds extract of Aglianico contained the higher amount of C (180 mg/g) respect to Merlot (100 mg/g) and Cabernet Sauvignon (70 mg/g).

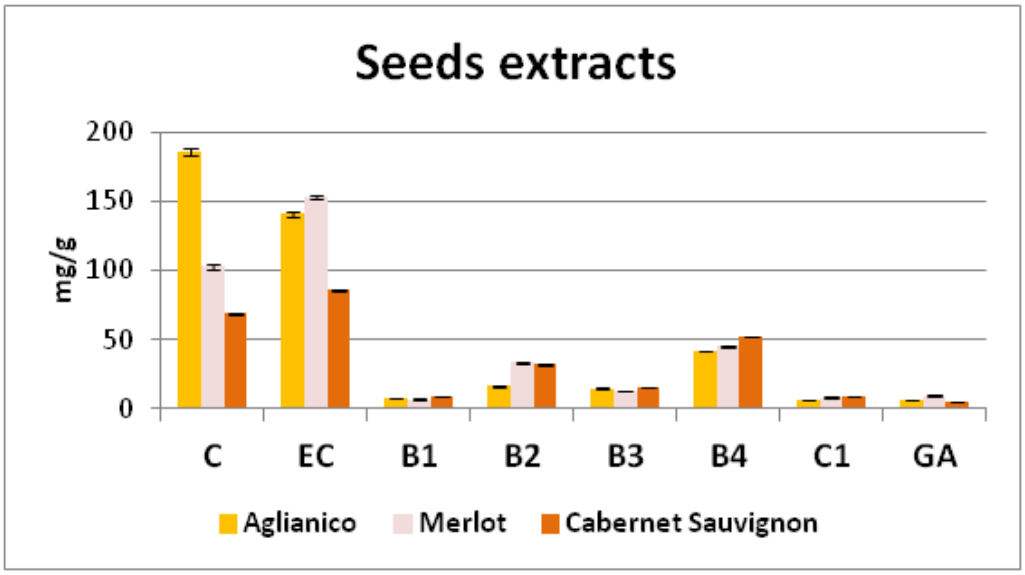

Fig. 2: Monomers (C-EC-GA), dimers (B1-B2-B3-B4) and oligomer (C1) content in seeds extracts of Aglianico, Merlot and Cabernet Sauvignon grapes.

As regard the content of EC, Merlot was richer (150 mg/g) than Aglianico (140 mg/g) and Cabernet Sauvignon (80 mg/g). Among dimers, the B1 and B4 were the most aboundant in Cabernet Sauvignon (9.5 and 52.5 mg/g, respectively). B2 content was similar between Merlot and Cabernet Sauvignon (about 33 mg/L), but lower in Aglianico (17 mg/g). B3 does not differ among cultivar. The trimer C1 was a little higher in Cabernet Sauvignon. Gallic acid, instead, was similar in Aglianico and Cabernet Sauvignon (about 6 mg/g) and in Merlot was present at a concentration of about 10 mg/g.

Generally, the concentrations of skins phenolics was much lower than in seeds extracts, as reported also by others with different cultivars (Fernandez *et al.*, 2007). In particular, a different distribution of phenolic compounds was observed (Fig.3). In fact, Merlot extract was characterized by a low content of C and EC (0.7 and 0.2 mg/g) respect to Aglianico and Cabernet S. (about 1.2 and 0.9 mg/g). In addition, B3 and C1 were absent in Merlot skins extract, while gallic acid (2.6 mg/g) was similar to Cabernet S. (2.5 mg/g) but higher than Aglianico (1.9 mg/g). The content of dimers B1, B2 and B4 was higher in Aglianico.

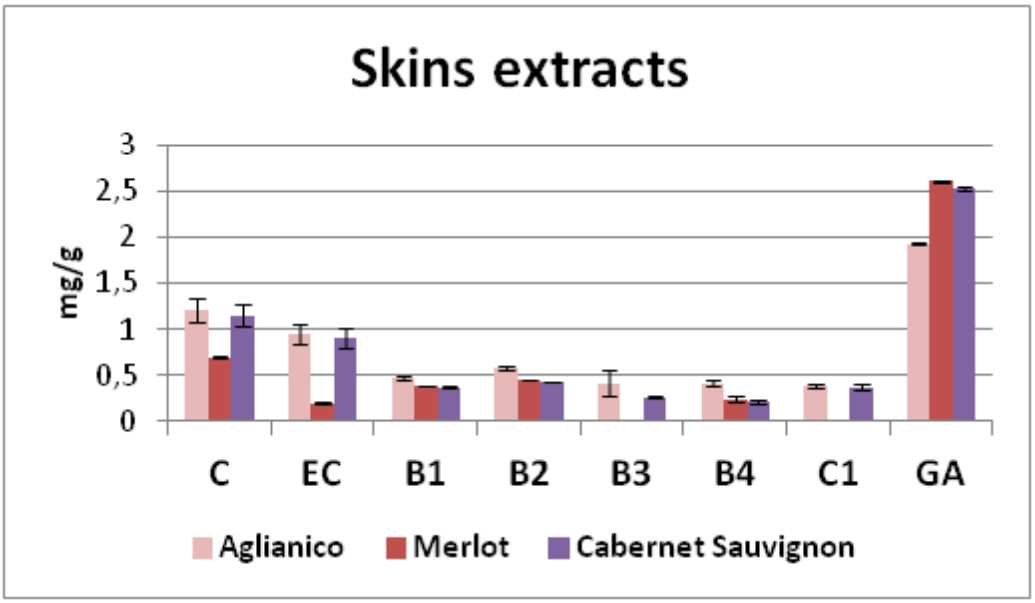

Fig. 3: Monomers (C-EC-GA), dimers (B1-B2-B3-B4) and oligomer (C1) content in skins extracts of Aglianico, Merlot and Cabernet Sauvignon grapes.

3.4.3.1.2 Olygomeric and polymeric fraction by acid-catalyzed depolymerization

In order to study the structural composition and characteristics of Aglianico proanthocyanidins, as well as of Merlot and Cabernet Sauvignon grapes, the acid-catalyzed depolymerization in the presence of phloroglucinol was carried out. It provided valuable information on proanthocyanidin subunit composition, mean degree of polymerization (mDP) of skins and seeds fractions. The mDP was calculated, as described by Drinkine *et al.*, (2007), briefly it is determined by dividing the sum of all proanthocyanidins subunits (flavan-3-olmonomers and flavanol-3-ol phloroglucinol adducts) to the sum of terminal units (flavan-3-ol monomers), in mole equivalents. The concentrations of flavan-3-ol monomers in grapes before the phloroglucinolysis reaction were subtracted from the corresponding concentrations of flavan-3-ols after reaction in order to determine the terminal subunits. The percentage of galloylation (%G) and the percentage of prodelphinidins (%P) as well as the mean degree of polymerization (mDP) of the proanthocyanidin fractions of seed and skin tannin extracts of Aglianico, Merlot and Cabernet Sauvignon are presented in Tab. 2.

Tab. 2: Structural composition and characteristics of Aglianico, Merlot and Cabernet Sauvignon seeds and skins monomeric/oligomeric (M/O) and polymeric (P) fractions.

P fraction				extention (%)				terminal (%)			
seeds	mDP	%G		C	EC	ECG		C	EC	ECG	
aglianico	5,6	24,8		6,5	73,4	20,1		29,2	24,2	46,7	
merlot	4,6	25,3		10,3	74,6	15,1		17,3	21,0	61,7	
cabernet sauvignon	5,8	21,0		8,6	80,2	11,2		18,6	14,3	67,1	
skins	mDP	%G	%P	C	EC	ECG	EGC	C	EC	ECG	EGC
aglianico	11,1	13,7	21,1	2,9	64,2	15,1	17,8	46,1	-	-	53,9
merlot	13,6	6,2	31,5	2,6	57,5	5,9	34,1	23,0	71,3	-	-
cabernet sauvignon	13,7	5,6	33,3	3,0	56,3	5,0	35,6	25,7	61,5	12,9	-
M/O fraction				extention (%)				terminal (%)			
seeds	mDP	%G		C	EC	ECG		C	EC	ECG	
aglianico	2,5	38,3		6,8	33,2	60,1		52,8	41,3	5,8	
merlot	1,9	17,6		15,9	56,7	27,3		37,8	55,3	6,9	
cabernet sauvignon	2,5	18,5		14,7	62,4	22,8		44,1	44,1	11,8	
skins	mDP	%G	%P	C	EC	ECG	EGC	C	EC	ECG	EGC
aglianico	3,8	11,9	11,6	4,9	71,5	11,5	12,2	60,0	17,0	12,9	10,1
merlot	2,3	12,0	23,8	2,5	40,4	14,6	42,5	87,9	3,4	8,8	-
cabernet sauvignon	2,0	10,4	26,0	3,6	30,6	13,8	52,0	86,5	5,1	8,4	-

This study represents the first structural characterization of Aglianico (A) grapes, in comparison with Merlot (M) and Cabernet Sauvignon (CS). In general, skin and seed proanthocyanidin profiles differed by their low amounts of galloylated derivates and higher mDP. These results are consistent with data concerning mDP values of polymeric proanthocyanidins in some publications, where values for grape seeds extracts ranged from 2.7 to 18.6 (Monagas *et al*., 2003; Fernandez *et al*., 2007) for other *V. Vinifera* varieties. However, literature data concerning mDP values of skin polymeric proanthocyanidins largely vary, from 11 to 83 approximately depending on the fractionation technique employed and the grape variety and vintage (Souquet *et al*., 1996; Sun *at al*., 1999). Polymeric and monomeric/oligomeric fractions of Aglianico skins and seeds were firstly characterized. Considering Aglianico, Merlot and Cabernet Sauvignon grapes of the Taburno DOCG geographical area, the mDP of polymeric fractions ranged from 11.1 of Aglianico to 13 for Merlot and Cabernet Sauvigon skins, while it was abot 4.7 for A and M, and 5.8 for CS seeds. It can be observed in Tab. 2 that the extension units of polymeric skins are composed of 2.9% C, 64.2% EC, 15,1% ECG, 17.8% EGC, while the terminal units of 46.1% C, no EC and ECG were detected, and 53.9% of EGC. The presence of a percentage of about 54% of EGC in the terminal units of A represents a rare instance because it was never found before. A high degree of prodelphinidin (%P) was observed for CS (33.5%) and M (31.5%), while A accounted for a 21.1%. As regard the polymeric seeds fraction, Aglianico showed the high percentage of ECG in extension units (20.1%) respect to Merlot (15.1%) and Cabernet S. (11.2%), while the contrary was observed in the terminal units: 46.7% for A, 61.7% for M and for CS. A higher degree of galloylation of 38.3% was also detected in Aglianico seed monomeric fraction than 17.6% of Merlot and 18.5% of Cabernet Sauvignon. This was mainly due to the presence in Aglianico of ECG as the extension subunit (60.1%), respect to Merlot (27.3%) and Cabernet Sauvignon (22.8%). A content of C of 6.8% and 52.8% characterized respectively the extension and terminal units of Aglianico monomeric seeds, quite different from Merlot and Cabernet Sauvignon (Tab.2). The monomeric/oligomeric fraction of Aglianico skins showed an higher mDP, a similar %G and low %P than the others. A high content of EC was observed in both extension and terminal units. Also in this fraction was detected the EGC as terminal units in Aglianico skins (10%).

3.4.3.1.3 Reactivity of phenolic fractions toward salivary proteins

The precipitation of salivary proteins by polyphenols is at the basis of the astringency phenomenon, and mainly depends on proanthocyanidin structural characteristics. It is very important to know the monomeric and oligomeric flavan-3-ols of the grape seeds and skins because they could contribute to the flavan-3-ol profile of the respective wines, and determine their gustative and mouthfeel properties. Also grape polymers contribute significantly to wine astringency (Sun *et al*., 2013), but the difficulty in the purification and the absence of standard references did not give us the possibility to quantify them. However, the monomeric/oligomeric and polymeric fractions of grapes were obtained by liquid/liquid solvent extraction for representing the totality of proanthocyanidins. In order to study the potential astringency of grapes, the monomeric/oligomeric and polymeric fractions of skins and seeds of Aglianico, Merlot and Cabernet Sauvignon cultivars were dissolved in model wine solution (ethanol 12%; pH=3.6; tartaric acid = 4 g/L) at a concentration of 1 g/L and analysed by SDS-PAGE-based method.

In Fig. 4 the electrophoretic pattern of human saliva (HS) before and after the interaction with the monomeric/oligomeric fraction of grape seeds (A) and skins (B) of Aglianico, Merlot and Cabernet Sauvignon was shown. The proteins from which the SPI was

calculated were at about 65 and 15 kDa. The Saliva Precipitation Index (SPI) revealed the reactivity of proanthocyanidins toward salivary proteins, and being correlated with sensory analysis, represents an evaluation of their astringency.
From the comparison between seeds and skins reactivity toward salivary proteins, it can be seen that the density reduction of bands after the interaction with seeds fractions was higher than skins fractions for all cultivars. The great capability of seeds to precipitate proteins respect to skins has also been shown (Gambuti *et al*., 2006).
The bands density reduction denoted that the Aglianico seeds fraction mainly precipitated proteins respect to Merlot and Cabernet Sauvignon.

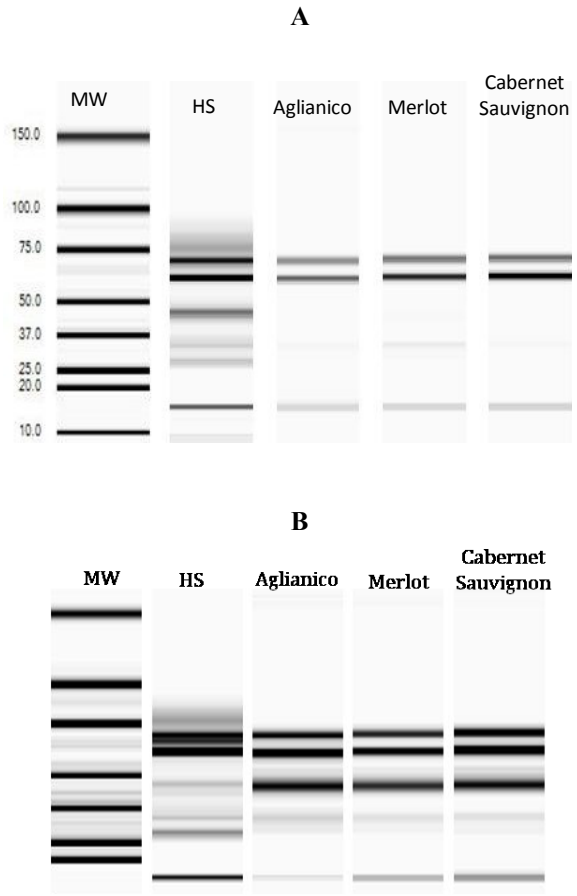

Fig. 4: Example of the SDS-PAGE electrophoresis of human saliva (HS) before and after the interaction with: Aglianico, Merlot and Cabernet Sauvignon monomeric/oligomeric seeds fractions (A), and skin fractions (B).

The SPI calculated by means of densitometric analysis for the monomers/oligomers and polymers of seeds fractions was shown in Fig. 5. Aglianico monomers/oligomers showed a higher value of SPI than polymers, while no differences between monomers/oligomers and polymers for Merlot and Cabernet Sauvignon were denoted. These values revealed that the astringency of Aglianico monomers/oligomers seeds was equivalent to about 1.9 g/L of gallic acid equivalent (GAE), respect to Merlot (1.7 g/L) and Cabernet Sauvignon (1.5 g/L). However, polymeric fractions were less astringent being egual to 1.66, 1.58 and 1.45 g/L of GAE for Aglianico, Merlot and Cabernet Sauvignon, respectively.

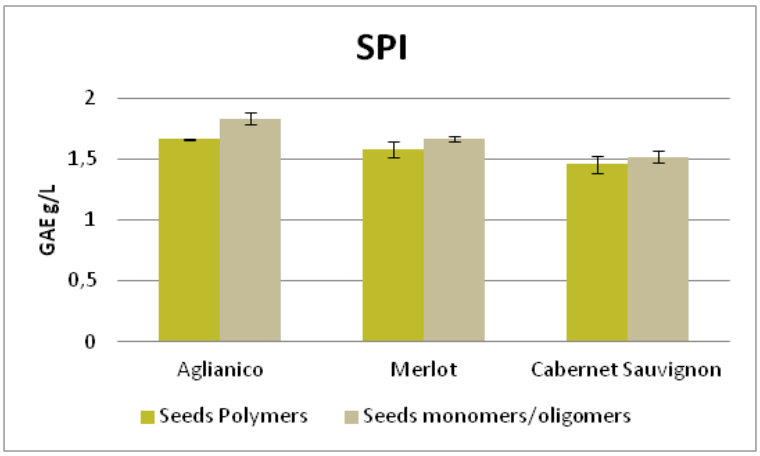

Fig. 5: the SPI (expressed as g/L of gallic acid equivalent GAE) of polymeric and monomeric/oligomeric fractions of Aglianico, Merlot and Cabernet Sauvignon

The SPI for skins extracts, as well as monomeric/oligomeric and polymeric fractions, of Aglianico, Merlot and Cabernet Sauvignon was determined (Fig. 6).

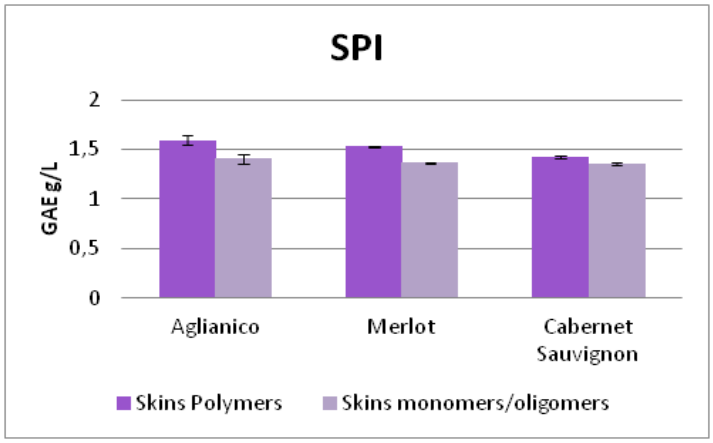

Fig. 6: The SPI (expressed as g/L of gallic acid equivalent GAE) of monomeric/oligomeric and polymeric skins fractions of Aglianico, Merlot and Cabernet Sauvignon grapes.

The reactivity toward salivary proteins of skins polymers was higher than monomers for all of the cultivars. The potential astringencies of monomers/oligomers were similar among cultivars. As regard the polymeric fraction the order of astringency based on the precipitation of salivary proteins, was Aglianico > Merlot > Cabernet. Surprisingly, Merlot grapes resulted more astringent than Cabernet Sauvignon, different from data reported by others (Chira *et al.*, 2009). However, it is also known that territorial and climatic parameters also influenced grape characteristics (Ubalde *et al.,* 2010). This great diversity in structural characteristics of Aglianico proanthocyanidins respect to Merlot and Cabernet Sauvignon reflected also the different reactivity toward salivary proteins, as

showed by the SPI. The high reactivity of Aglianico grapes, both seeds and skins, make this cultivar a good model for astringency.
In order to understand the relationships among grape composition and astringency, expressed as the ability of proanthocyanidins to precipitate salivary proteins, a statistical analysis that takes into account all of these variables was carried out. Each structural parameter was correlated with the SPI, as shown in Tab.3.

Tab. 3: Correlation value between structural parameters and SPI, and significative values.

	SPI	p-value	
mDP	-0,1565	0,6272	
%G	0,8446	0,0005	***
%P	-0,5835	0,0464	*
eC%	0,3770	0,2270	
eEC%	-0,0444	0,8910	
eECG%	0,8167	0,0012	**
tC%	0,1591	0,6213	
tEC%	-0,3326	0,2908	
tECG	-0,1502	0,6413	
eEGC%	-0,6669	0,0179	*
tEGC%	0,0792	0,8067	
significativity	p <	0,05	*
		0,01	**
		0,001	***

Based on the correlation analysis, it can be observed that the precipitation of salivary proteins (SPI) was positively and significantly correlated with %G (R = 0.844) and eECG% (R = 0.817) while is negatively correlated with eEGC% (R = -0.667) and %P (R = -0.583). However, the variables that contributing the most to the predictive model of SPI with a R^2= 0.9119 were mDP, %G and eECG. When the variables are used for a multiple linear regression analysis (Fig. 7), they explained 99.9 % of the SPI variance; the levels of ECG extension units (eECG%) explained 13.3%, the galloylation content (%G) account 42.8%, whereas mDP justified 43.8% of the total SPI variance.

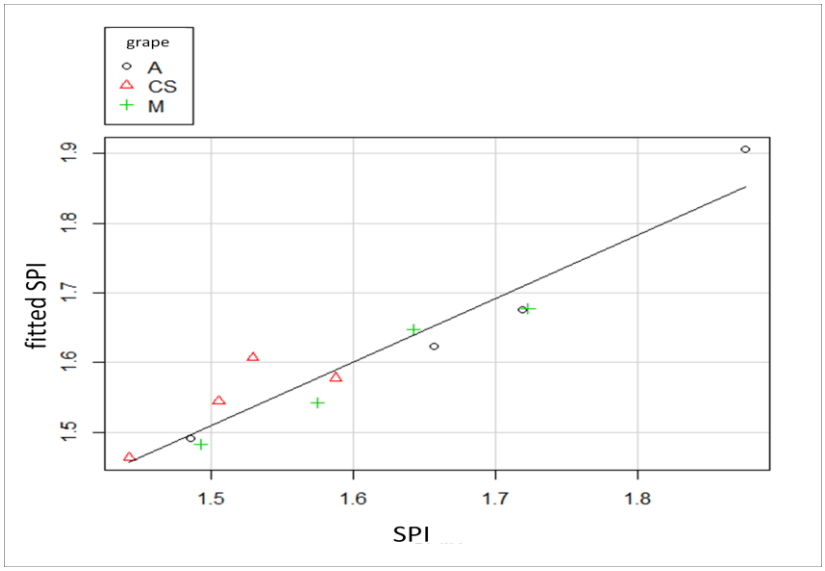

Fig. 7: Prediction of SPI from the structural composition of grapes Aglianico (A), Merlot (M) and Cabernet Sauvignon (CS).

The importance of these variables can be also seen in the predicted model:

$$SPI = 1.28 - 0.013x(mDP) + 0.001x(\%G) - 0.003x\ (eECG\%)$$

These results confirm the importance of mDP and %G in the astringency mechanism, but stresses also that the percentage of ECG in extension units of grape contribute significantly to the precipitation of tannins with salivary proteins.

3.4.4 Conclusions

For the first time the characterization of proanthocyanidins of seeds and skins of Aglianico grape cultivar was carried out. A comparison with international cultivar as Merlot and Cabernet Sauvignon was made in order to understand the peculiarities that make Aglianico wine different from a sensorial point of view from others. In fact, Aglianico is considered more astringent over the average of red wines. This may due to the chemical composition of grapes. In particular, it has been shown that the precipitation of salivary proteins, which is at the basis of the astringent sensation, was mainly due to the percentage of galloylation, the presence in the extension units of ECG, and negatively correlated with the mean degree of polymerization. This study puts the bases for better understand how to manage the vinification processes with grape cultivar rich in polyphenols as Aglianico, and in particular with seeds characteristics that may compromise the sensory quality of wine.

3.4.5 References

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APPENDIX 1

LIST OF INTERNATIONAL JOURNAL PUBLICATIONS:

1. **Rinaldi, A**., Gambuti, A., Moine-Ledoux, V., & L. Moio, 2010. Evaluation of the astringency of commercial tannins by means of the SDS–PAGE-based method. Food Chem 122: 951–956.
2. Gambuti, A., **Rinaldi, A**., Pessina, R., Lisanti, M.T., & L. Moio, 2011. Partial dealcoholisation of red wines by membrane contactor technique: influence on colour, phenolic compounds and saliva precipitation index. Eur Food ResTech. 233, 4:647-655.
3. **Rinaldi A.,** Gambuti A., Moio L. 2012. Precipitation of Salivary Proteins After the Interaction with Wine: The Effect of Ethanol, pH, Fructose, and Mannoproteins. J Food Sci. Apr; 77(4):C485-90. doi: 10.1111/j.1750-3841.2012.02639.x
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5. Gambuti A, **Rinaldi A**., Moio L. 2012. Use of patatin, a protein extracted from potato, as alternative to animal proteins in fining of red wine. Eur Food Res Tech. Volume 235, Issue 4, 753-765.
6. Gambuti A, **Rinaldi A**, Ugliano M, Moio L. 2012. Evolution of Phenolic Compounds and Astringency during Aging of Red Wine: Effect of Oxygen Exposure before and after Bottling. J Agr Food Chem. dx.doi.org/10.1021/jf302822b.
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LIST OF CONGRESS PROCEEDINGS PUBLICATIONS:

1) **Rinaldi A**., Gambuti A., & Moio L. 2011. Astringency and precipitation of salivary proteins: the effect of ethanol, pH, fructose and mannoproteins. Wine Active Compounds 2011. Proceedings of the Second Edition of the International Conference series on Wine Active Compounds. 24-26 March, Beaune, France. 209-210.
2) **Rinaldi A**., Gambuti A., & Moio L. 2011. Application of the SPI (Saliva Precipitation Index) to evaluate the effect of fining agents on Aglianico (*Vitis Vinifera* cv.) wine. 9ème Symposium International d'Oenologie de Bordeaux, 15-17 Juin, 2011, Bordeaux, France.
3) **Rinaldi A**., Gambuti A., & Moio L. 2011. Reduction of tannins reactive towards salivary proteins by different *Saccharomyces cerevisiae* yeast strains during alcoholic fermentation. 9ème Symposium International d'Oenologie de Bordeaux, 15-17 Juin, 2011, Bordeaux, France.
4) Gambuti, A. **Rinaldi A.**, & L. Moio. Patatin, a protein extracted from potato used as alternative to animal proteins in wine fining. XXXIV World Congress Vine and Wine OIV, 20-27 June 2011. Porto, Portugal.
5) **Rinaldi A**., Gambuti A., & Moio L. 2011. Astringency and precipitation of salivary proteins: the effect of ethanol, pH, fructose and mannoproteins. Proceedings of 16° Workshop on the Developments in the Italian PhD research on Food Science Technology and Biotechnology. 21-23 September, Lodi, Italy. 267-268.

APPENDIX 2

ORAL COMMUNICATIONS:

1. **Rinaldi A**., Gambuti A., & Moio L. 2011. Astringency and precipitation of salivary proteins: the effect of ethanol, pH, fructose and mannoproteins. Wine Active Compounds 2011. 24-26 March, Beaune, France.
2. **Rinaldi A**., Gambuti A., & Moio L. 2011. Application of the SPI (Saliva Precipitation Index) to evaluate the effect of fining agents on Aglianico (*Vitis Vinifera* cv.) wine. 9ème Symposium International d'Oenologie de Bordeaux, 15-17 Juin, 2011, Bordeaux, France.
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POSTERS:

1. **Rinaldi A**., Gambuti A., & Moio L. 2011. Reduction of tannins reactive towards salivary proteins by different *Saccharomyces cerevisiae* yeast strains during alcoholic fermentation. 9ème Symposium International d'Oenologie de Bordeaux, 15-17 Juin, 2011, Bordeaux, France.
2. **Rinaldi A**., Gambuti A., & Moio L. 2011. Astringency and precipitation of salivary proteins: the effect of ethanol, pH, fructose and mannoproteins. Proceedings of 16° Workshop on the Developments in the Italian PhD research on Food Science Technology and Biotechnology. 21-23 September, Lodi, Italy. 267-268.
3. **Rinaldi A**., Gambuti A., & Moio L. 2012. Application of the SPI (Saliva Precipitation Index) to the evaluation of red wine astringency. Chimalsi, IX Congresso Italiano di Chimica degli Alimenti "Alimenti, Alimenti Funzionali e Nutraceutici: Aspetti Chimici, Biologici e Tecnologici", 3 al 7 Giugno 2012, Ischia.
4. Gambuti A., **Rinaldi A**., & Moio L. 2012. Evolution of Phenolic Compounds and Astringency during Aging of Red Wine: Effect of Oxygen Exposure before and after Bottling. Chimalsi, IX Congresso Italiano di Chimica degli Alimenti "Alimenti, Alimenti Funzionali e Nutraceutici: Aspetti Chimici, Biologici e Tecnologici", 3 al 7 Giugno 2012, Ischia.

5. **Rinaldi A**., Gambuti A., & Moio L. 2012. Application of the SPI (Saliva Precipitation Index) to the evaluation of red wine astringency. XXXV World Congress Vine and Wine OIV, 18-22 Juin 2012 Izmir (Turchia).
6. **Rinaldi A**., Gambuti A., & Moio L. 2012. The enological potentiality of patatin, a protein extracted from potato, as not-allergenic fining agent of musts and wines. XXXV World Congress Vine and Wine OIV, 18-22 Juin 2012, Izmir (Turchia).
7. Gambuti A., Siani T., Erbaggio A., **Rinaldi A**., Frusciante L., Bonfante A., Albrizio R., Basile A., Buonomo R., De Mascellis R., Giorio P,. Guida G., Agrillo A., Manna P. Terribile F., Moio L. 2012. Influence of soil-plant water stress over technological and phenolic maturation of cv Aglianico (*Vitis Vinifera* L.) grape. XXXV World Congress Vine and Wine OIV, 18-22 Juin 2012, Izmir (Turchia).
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